

nature

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Opening up Government

THE much disliked Section 2 of the Official Secrets Act seems at long last to be on its way out. A White Paper just published by the British government (Cmnd. 7285; HMSO, 70p) responds to the six-year-old report of the Franks Committee by agreeing in large part with the recommendations made by that committee and proposing a new Official Information Act. But the title of this act is somewhat misleading; it refers to restrictions on information available, not to making more information accessible. Those who looked for something akin to the US Freedom of Information Act are going to be disappointed.

Section 1 of the 1911 Act deals with the transmission of information that may be useful to an enemy. Section 2, however, is a much vaguer affair making it an offence for civil servants (and some others) to pass on without authority information received because of their official position. For every one instance where it makes perfect sense to discourage the passing on of information there is another where the restriction is preposterous. As the White Paper puts it "there must be hundreds of technical contraventions of the Act every day as Crown servants tell friends or relatives trivial details about their work." In practice commonsense generally prevails, but not unnaturally the civil servant tends to err on the side of caution.

What Franks recommended, and what the government seems set to do, is to restrict dramatically, in an Official Information Act, the scope of the information the release of which would continue to be a criminal offence. The Act would also make it much clearer what information fell within this category. In the area of law and order any information which could assist the committing of illegal acts, regardless of whether it had a classification, would be protected. Likewise any information held by the government about private individuals and concerns, would again be protected, regardless of any classification. On the other hand information in the fields of defence, internal security and foreign relations would be protected only if it bore a classification. And information in other fields should not be protected at all.

That six years have elapsed since Franks reported before the government felt able to make a move must mean that these reforms have been resisted by some

powerful people. But even so many concerned with gathering news and information have expressed bitter disappointment that more has not been said about 'open government'. The 1911 Act had nothing specifically to say on this subject, although undoubtedly it helped create an environment in which civil servants did not feel they could be entirely open. In recent years, the White Paper points out, government has indeed become more open with Green Papers, Select Committees, an Ombudsman and various other administrative measures. But should we go further and have some sort of Freedom of Information Act which would open up much more of the paperwork of government to the outsider? The White Paper lamely concludes that more studies need to be carried out before this question can be answered and this has drawn predictable flak from many who wonder why it has taken six years to discover that. This reaction, justifiable as it is, should not obscure, however, the central question of what would serve open government best.

An Official Information Act clarifies the civil servant's position but it does relatively little to encourage him to talk freely about what he is predisposed to keep quiet. A Freedom of Information Act would certainly allow outsiders access to papers, but they would have to know what to ask for. Without prompting from the civil servant some very important documents, theoretically accessible, would never be requested. Further, civil servants and others who thought their work likely to be exposed to all and sundry would be much more non-committal in documents and much more prone to make key observations verbally or on bits of paper that never got into the records. So it is at least arguable that open government would not best be served by a Freedom of Information Act. What is more needed is a gradual development of confidence between government, press and public that serious matters will be treated seriously and that information passed on will be complete. This requires a new breed of politician and a new breed of civil servant who trust the public and recognise that a 'public debate' on a subject means hearing the views of more than the charmed few. It also needs the sort of journalist who can respond sensibly to more openness. We really need new attitudes, not new acts. □



US widens the options on fusion research

David Dickson reports from Washington on new doubts about the future of the Tokamak

CONCERN at the potential engineering problems facing the development of nuclear fusion reactors based on the tokamak design is leading the US to widen the range of its search for potential fusion technologies.

Although the tokamak design is at present the closest to demonstrating its scientific feasibility—the design is also the basis of the Joint European Torus (JET) to be built by the EEC at Culham in the UK—scientists also fear that its engineering problems may prove to be the most complex.

Magnetic mirrors

In order to hold other options open, the US Department of Energy is contemplating holding back plans for further rapid expansion of the tokamak programme, and at the same time increasing the support that it provides for alternative fusions design concepts, in particular the use of magnetic mirrors for confining the superheated plasma.

The department is also reviewing a range of more speculative proposals, each of which is presently at little more than the theoretical stage, with a view to selecting two or three for increased funding. This would bring the work to a stage at which the potential commercial viability could be assessed against the more conventional approaches.

Ironically, one of the strong contenders for additional support is a magnetic confinement scheme being developed as the Los Alamos scientific laboratory using a method known as the 'reverse field pinch'. A configuration first noted—but at the time not properly understood—by British scientists using the UK Atomic Energy Authority's ZETA reactor at Harwell in the 1950s.

As fusion power rapidly approaches the first demonstrations of its scientific feasibility — Princeton University's Tokamak Fusion Test Reactor is due to begin operation in 1981, with intertial confinement at Los Alamos not far behind—congressional enthusiasm for promises of a clean, renewable source of energy is currently running high.

Last week, for example, the House of Representatives added \$22.9 million to the Department of Energy's request for fusion research in 1980, bringing the total to almost \$225 million. Of this figure, \$8 million is to establish a civilian research programme into the potential commercial exploitation of intertial confinement systems. At present all such work is being carried out in a military context because of the

laser's capacity for triggering nuclear weapons.

The house also added \$7 million to fund studies of a highfield, scaled-down version of the tokamak, an idea being pushed by a firm called Inesco which, it is claimed, promises to provide commercial fusion power 10 to 15 years sooner than current fusion plans.

But if congressional representatives, many of whom reflect the growing interest in fusion technology of high technology companies such as the Grumman Corporation, are keen to press ahead quickly to the commercialisation stage, the administration is pausing to take a look at its priorities, attempting to make sure that the choices lying ahead are taken correctly.

Critical reports

Two recent reports have reflected this thinking. The first is a report prepared for the Office of Science and Technology Policy on basic research in the Department of Energy, which criticised attempts in both the magnetic confinement and intertial confinement fusion programmes to "move ahead too rapidly without adequate theoretical, experimental and engineering assessment of existing results".

The second is a more detailed report on fusion research prepared by a group chaired by Dr John S. Foster, Vice-President for Research and Development at TRW Inc., at the request of Dr John Deutch, Director of the Office of Energy Research in the Department.

This report expresses particular con-

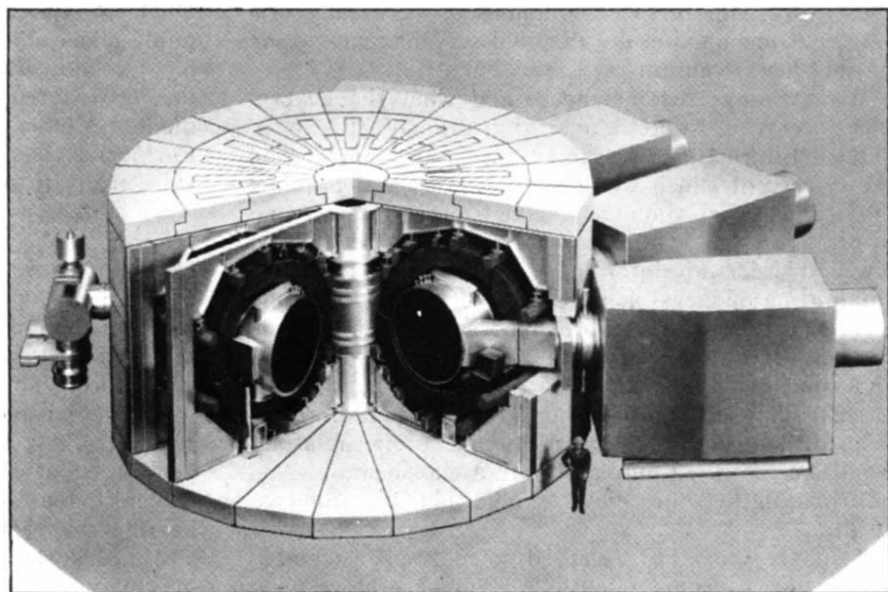
cern about the present heavy concentration of funds on experiments using tokamaks. The group claims that this strategy "places undue emphasis on a single approach, which constitutes an unnecessarily high risk" and suggests increased efforts in other areas to "allow a realistic choice to be made from among several hopefully viable alternatives".

The group says that although tokamaks are currently the most advanced scientifically, they seem on the surface to be the most complex of the possible alternative approaches to fusion from the standpoint of converting into an energy producer. It suggests that the present profusion of tokamaks is probably the result of a "bandwagon effect".

As to the intertial confinement technique, the report says that although here again demonstration of scientific feasibility is close, the programme so far has lacked adequate emphasis on the development of driver mechanisms capable of providing the system energy efficiency (the ratio of the energy in to the energy out) necessary for practical energy source.

Appearing just at the time when preliminary budget proposals for 1980 are being prepared for submission by government agencies to the Office of Management and Budget, these two reports have provided legitimation for the re-ordering of priorities for fusion research within the Department of Energy.

At one point, indeed, one of a range of proposals under consideration was to cut back severely funds for the magnetic fusion programme, diverting the funds to more long-range projects. Fol-



Princeton's Tokamak Fusion Test Reactor is due to go into operation in 1981.

lowing sharp protests from Congress and elsewhere, however, this option has been discarded, and it looks as if funding for the magnetic fusion programme, although not as high as some would like to see, will be maintained at a reasonable level, although with a slight shift in emphasis from the tokamak projects to those involving mirror confinement.

At the same time the department is looking closely at other, less conventional schemes. Currently about 12 of these are being funded by the department—out of the 100 or so proposals which are received each year—at an annual cost of about \$15 million.

These various schemes are now being evaluated both from the scientific and the technological points of view, with the intention of selecting the two or three most promising to receive significant additional funding.

More or fewer problems?

At present, these various alternatives are so far behind the more main-line schemes that some are sceptical whether they can, in fact, ever catch up sufficiently to compete realistically. Others, however, feel that such additional funding could provide a breakthrough to a new system presenting fewer problems than those now being considered.

"We want to broaden the programme and develop alternatives to tokamak. I don't think anybody can say which approach will work the best, Dr Deutch said last week. "It's a matter of keeping the door open to new ideas," said another department official.

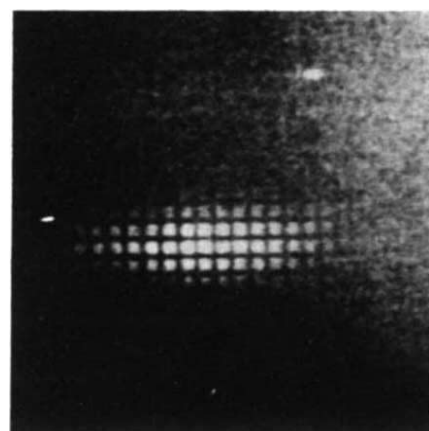
One result of holding back from putting all the research eggs into one basket—the lessons of which the administration has learnt from its experiences with the Clinch River Fast breeder—is that it could lengthen the timescale for the commercialisation of fusion energy. Dr Deutch is now predicting that the first commercial fusion reactor will be in operation by 2005, and the second ten years after that.

In Europe, energy officials have been taking careful note of the two US reports. But, if it were necessary, it would not be an easy matter to shift the EEC five-year research programmes, which run from 1975–80. Mirror machines, of which there were many a decade ago in Europe, have been abandoned, and the commitment to JET is substantial.

The commissioner responsible for JET, M. Reginald Saison, said this week that "we are reassessing the technological problems". But he could not say if research funds would be shifted towards engineering and away from pure confinement research. "The matter is under discussion".



PETRA'S FIRST LIGHT: the first synchrotron light from PETRA, Germany's brand new accelerator, causes the physicists to celebrate. Centre, Professor Gustav-Adolf Voss, leader of the project. PETRA has already stored a 3 milliamp, 5 GeV electron beam for 2 hours, and accelerated the beam from 5 to 8 GeV. This week it should accept positrons in the opposite direction and produce its first collisions. Commissioning is going "extremely well" says Voss, with the machine behaving almost exactly as predicted.



NIH relaxes recombinant DNA guidelines

A revised version of the guidelines for conducting research involving recombinant DNA techniques, in which the safety precautions required for a range of experiments would be substantially reduced, was due to be published this week by the US Department of Health, Education and Welfare.

The guidelines have been prepared by Dr Donald Fredrickson, director of the National Institutes of Health, following extensive discussion in both the scientific and the lay community, and taking into account the knowledge that has been gained about the use of the techniques since the existing guidelines were first published in June 1976.

In the light of this new knowledge, the revised guidelines are expected both to relax the safety requirements for certain types of experiments, and exempt others from the need for regulation.

It is also expected that the new draft will compromise on an earlier suggestion that local biohazard committees be set up with the power to give the go-ahead to recombinant DNA experiments performed within the guidelines, merely supplying NIH with formal notification.

Following concern that such a system could lead to variations in interpretation of the guidelines, it is now

expected that NIH approval will, as at present, still be required before new projects can go ahead, but that the local committees will be able to renew approval for such projects.

The draft version of the revised guidelines will be open for public discussion for a period of 60 days, after which a final version will be prepared and promulgated by Dr Fredrickson. Mr Joseph Califano, secretary of HEW, says in a preamble to the guidelines that he is particularly keen to receive comments on their non-technical aspects, and a public hearing is to take place towards the end of the comment period, i.e. in mid-September.

Meanwhile in the House of Representatives, a bill sponsored by Representatives Harley Staggers and Paul Rogers to extend the NIH guidelines to cover all experiments involving recombinant DNA in both the public and private sector, of which little has been heard since March, is now expected to be debated on the floor of the House within the next few weeks.

But in the possible absence of any legislation, NIH is contemplating offering both a registration and a host-vector certification service to private companies as the basis of a system of voluntary compliance.

David Dickson

Brown pelican threat to space shuttle

MAJOR alterations may be required in the flight path of the US space shuttle delivery system, whose first flight is due next summer. This follows the discovery that its planned descent route could threaten the habitat of an officially endangered species, the brown pelican.

The only significant breeding grounds of the brown pelican on the west coast of the US are on the Anacapa Islands, 20 miles off the coast of southern California. On present plans, the space shuttle will pass over the islands—still at supersonic speed—on its descent to the Edwards Air Force Base, north of Los Angeles.

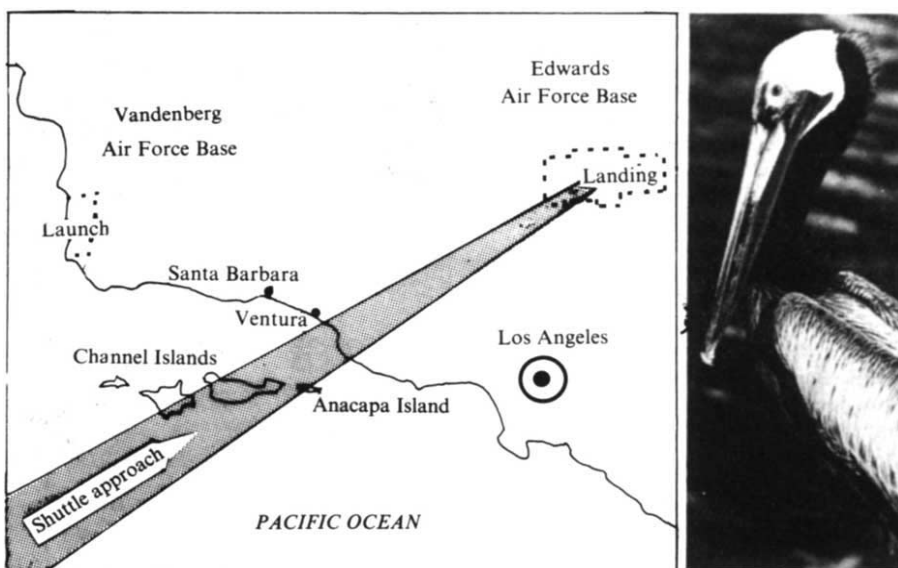
Studies are now being carried out by the biology department at the University of San Diego and sponsored by the US Air Force, which is responsible for the shuttle delivery system, on the effect of noise disturbance on the brown pelican's breeding habits. The Department of the Interior's Fish and Wildlife Service will then decide whether it considers the space shuttle will jeopardise the survival of the brown pelican.

As the law stands at present, a ruling that the space shuttle did pose a threat would require the Air Force to change its plans, possibly by altering the descent flight path. In a letter to Senator John Culver of Iowa earlier this year, the Fish and Wildlife Service said that the space shuttle was one of a number of projects which posed "potential consultation problems".

However under an amendment to the 1973 Endangered Species Act introduced by Senator Culver and accepted by the Senate last week, with the strong possibility of becoming law by mid-autumn, cases where the Department of the Interior and another government agency cannot agree on steps to preserve an endangered species could be referred to a seven-person arbitration committee.

Moves to set up such a committee, which would have power to exempt certain projects from the provisions of the Act, follow criticism of the Act's inflexibility. This was illustrated most dramatically in last month's decision by the Supreme Court that the Tennessee Valley Authority could not complete the multi-million dollar Tellico Dam as this threatened to extinguish a three-inch species of fish, the snail darter.

In agreeing to extend the En-



dangered Species Act for a further three years, the Senate rejected a stronger amendment under which any restrictions on a federal project in order to protect an endangered species would have to be justified on social and economic grounds. However it also rejected pleas from both environmentalists and the administration that the Act should be left intact, and that any weakening would seriously undermine its usefulness.

If, as seems likely, a similar amendment is accepted by the House of Representatives, the amendment could be signed into law by President Carter within a few months. The first case for consideration by the committee could well be the snail darter; but the brown pelican is also among potential candidates for consideration.

Precarious population

Recent measures to protect the brown pelican on the East Coast, and in particular on the coast of Florida, have reversed the decline in population. But on the West Coast the future of the subspecies known as the California brown pelican remains precarious.

This year, for example, although the colony on Anacapa Island includes several hundred pairs of birds, no eggs have been hatched, possibly due to a shortage in fish supply. One Fish and Wildlife Service official said last week that the species was "hanging on by its fingernails in California".

Two aspects of the space shuttle flight path are causing concern. The first is the pressure of the supersonic boom, which could cause the eggs to crack. The second is the disturbance to the pelicans during the nesting season—which normally extends from February into July. However, the Air Force could argue that, with a reason-

able number of brown pelicans now enjoying stable existence elsewhere in the US, as well as major breeding grounds on the Pacific coast of Mexico, the space shuttle disturbances would not put the whole species at risk.

Such an argument could well receive a more sympathetic hearing from an arbitration committee, able to bring broader social and economic factors into consideration, than by the Department of the Interior, whose judgement on the potential threat of a project would be based on biological and ecological grounds.

David Dickson

● A warning that the intrusion of human activities into the natural environment, in particular the increasing destruction of large areas of tropical rain forest, could lead to the disappearance of hundreds of thousands of unique and irreplaceable life-forms is given in a report published last week in Washington by the Worldwatch Institute.

Less than half—and possibly as few as 15%—of the world's natural species have been recorded in the scientific literature, according to Erik Eckholm, author of the report *Disappearing species: the social challenge*. He adds: "If current patterns of human activity continue, a good share of the unrecorded majority of species will vanish before their existence, much less their biological importance or economic utility, is known."

He suggests a massive expansion of the UNESCO Biosphere Reserve system. So far, 144 Biosphere Reserves have been established in 35 countries, but tropical ecosystems are badly unrepresented. Mr Eckholm recommends a strategy by which nature-reserve needs are identified country by country, and governments then encouraged to establish them. □

The secret is to have no secrets

EARLIER this month, Anatolii Shcharanskii received a sentence of three years' imprisonment plus ten years' exile for divulging information officially classified by the Soviet authorities as "secret". According to the rumours routinely described as "reliable diplomatic sources", it would appear that much of this secret information was in fact a denial of secrecy. In his capacity as a human rights activist, Shcharanskii regularly brought to the notice of the foreign press details of Jewish scientists refused emigration visas on the grounds that they had been engaged in "secret" research.

The scientists, and Shcharanskii as their spokesman, fervently denied that their own work, or that of the institutes where they worked, was militarily sensitive. Nevertheless, to Soviet officialdom, it would appear that even to discuss matters of secrecy or otherwise is itself a breach of national security.

The "secrecy" of research is an argument frequently advanced by the Soviet authorities to explain why some Jewish scientist has not been allowed to emigrate to Israel. Since the notorious "education tax" on would-be emigrants was allowed to lapse some years ago, so that a departing scientist no longer had to repay the notional cost of his higher education, "secrecy" has been invoked so regularly as a barrier to emigration that it could appear to

the outside observer that virtually all Soviet research may be classified as "secret".

Indeed, some statements by the Soviet authorities seem to point in that direction. When Vladimir Slepak, a *refusnik* cyberneticist, protested that his work could not possibly be secret, since the Soviet Union was at least five years behind the USA, it was explained to him that "that precisely is the secret".

During the 1976 Revolution Day celebrations, Moscow Radio claimed that it was meaningless for western campaigners to claim that such-and-



"I hear she wants to emigrate."

such scientist had no access to secrets "since only we know what our secrets are"—and when no Western protests were forthcoming to what had seemed simply a routine propaganda broadcast, it was then intimated to Corresponding-Academician Veniamin Levich that the West had clearly concurred with the Soviet Union on this point!

Access to military secrets is, indeed, considered by all human rights legislation a valid impediment to emigration. Normally, however, a reasonable term is set, after which the "secrets" are assumed to be obsolete or generally known. Some five to seven years would appear standard in most countries. The Soviet Union, however, sets far higher limits. Recently, Irina Brailovskaya (a mathematician) was told that her term of secrecy was for 30 years, of which five had already expired, while Aleksandr Lerner, a cyberneticist, was informed that his was "for life".

Normally, however, would-be emigrants not only are not told of how long they must wait for security clearance; they do not even have any means of knowing, short of applying for a visa, whether or not they are eligible to emigrate at all. Since application to emigrate routinely results in dismissal from one's post, for those whose jobs are so secret that they themselves are unaware of the secrecy, request for a visa can result in several years expiating their "secret" knowledge in the jobless limbo of the *refusnik*.

Vera Rich

Tungus expert discovers biofields

ALEKSEI ZOLOTOV must surely be the supreme example of the heights of scientific attainment which the ordinary citizen of the Soviet Union can achieve. Originally, it would seem, merely an oil-driller, he was coopted, at some time during the mid 1960's, on to one of the geo/astrophysical expeditions to determine the origin of the Tunguska phenomenon. He has since become something of a Tunguska phenomenon himself.

Originally, Zolotov was merely an "expert" on local conditions and terrain. Gradually, however, he somehow became accepted as an expert on the phenomenon itself, propounding his theory that the mysterious body which caused such widespread devastation of the tundra was an extra-terrestrial space-craft which came to grief, disintegrating into a nuclear explosion.

Astrophysics, however, it would appear, is not Zolotov's sole interest. He has now ventured into biophysics, and, according to a recent TASS announcement, has postulated that, in addition to the "generally accepted" electromagnetic and gravitational fields,

there also exists a "biofield", research into which could "have an enormous influence on the development of science, in particular medicine".

In defence of his hypothesis, Zolotov claims that he can slow down the action of a chronometer "using the biofield of his hands". "Biofield", he says, is a random gift, like musical or mathematical talent; Zolotov himself, unfortunately, is only moderately gifted—otherwise he might be able to stop the chronometer entirely.

His talents, however, lie more in the realm of investigation. Already he claims to have observed that the biofield can affect a crystal oscillator. It is possible, he says, to photograph the "biofield", using the fact that light in such a field is dispersed. As a result of these investigations, he claims to have established, for example, that an "ailing thyroid gland" produces a biofield "in the shape of a dagger some 2 to 3 metres long". A healthy person sensitive to "biofields" can therefore, says Zolotov, restore normal functioning to the gland by influencing its biofield by his own.

None of the phenomena related are particularly new to investigators of the paranormal. The Kirlians were producing photographs, some two decades ago, which were claimed to show the energy fields of living organisms (and which, in the case of human subjects, showed "flares" at the traditional acupuncture points).

What is most remarkable about this latest claim of Zolotov is the support he regularly gets from TASS, first for his UFO/nuclear explanation of Tunguska, and now for his "biofield". Parapsychology, and its related phenomena, have always been a valid field of research in the Soviet Union, which has a vested atheistic interest in proving a physical basis for any phenomena which might otherwise be attributed to a nonmaterial "soul".

Nevertheless, one can only sympathise with Dr Peter Zolotov, Director of the Kirillin branch of the All-Union Geophysical Institute, where Zolotov officially works. Asked to comment on his subordinated work, Brodskii gave a non-committal answer. Zolotov's research he said, "seems to be interesting and promising".

Vera Rich

Why send probes to Venus?

Professor **Donald Hunter** of the University of Arizona tells what next week's Venus launch may achieve

THE Pioneer Venus Orbiter is well on its way, after a perfect launch on 20 May. If all goes well, early on the morning of 7 August it will be followed by the Multiprobe. These are actually two separate missions, telescoped together by the mundane realities of obtaining funds. Originally, they were supposed to go at successive opportunities, but various budgetary delays caused them to be flown the same year. The Orbiter takes a longer and somewhat slower path; both arrive in early December, about five days apart.

Venus and Earth seem as if they should be twins; their mass and radius are almost identical; although Venus gets twice the solar flux, it actually absorbs nearly the same amount of heat because it reflects a larger fraction. Instead, on Venus, we find a planetwide cloud of H_2SO_4 droplets and a uniform surface temperature of about 750 K at the bottom of 90 atm of CO_2 . These ingredients also exist on Earth, but the CO_2 is nearly all buried as limestone, and the sulphur too is mostly buried. HCl in the stratosphere suppresses all detectable signs of an ozone layer. Many of these differences are traceable to the presence or absence of liquid water; it seems that Venus either never had much, or somehow lost it. The other differences are reminiscent of what we are now doing to our own planet—releasing large amounts of SO_2 from industrial processes; building up the CO_2 level of the atmosphere through combustion of fossil fuels; and releasing chlorinated hydrocarbons at an ever-increasing rate. In truth, we are not likely to turn Earth into another Venus, but a better knowledge of Venus should give us a better chance to assess the consequences of our current actions.

The ideas are examples of comparative planetology applied to practical concerns. There are many additional areas whose interest is mainly intellectual, for example:

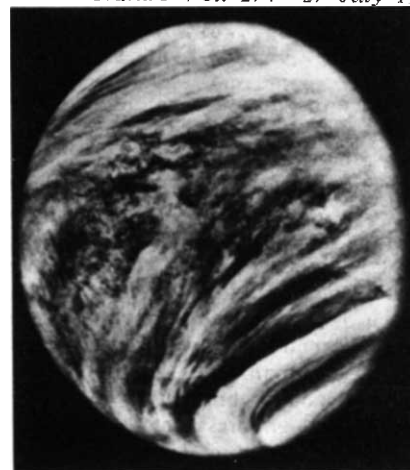
- Study of the upper atmosphere and the ionosphere and their effect of and on the solar wind (similar things may happen occasionally on Earth when its protective magnetic field is temporarily absent during a reversal.)
- The meteorology of a planet very different from our own. Venus rotates very slowly, and atmospheric motions are also slow, except near the cloud tops where there is a steady 100 m/s wind in the same retrograde direction as the rotation.

- Mapping of surface features to permit study of geological processes.

How do the Pioneer Venus missions contribute to these fields and how do they fit into the (presumably) continuing program of the USSR? The latter question can only be answered in retrospect, since the payloads are known only for past missions. However, the main emphasis of recent Soviet studies has been on surface measurements, whereas Pioneer Venus concentrates on the atmosphere, apart from the radar mapper on the Orbiter.

The Pioneer Venus Multiprobe mission consists of 5 separate vehicles which travel together to the vicinity of Venus. The 4 probes enter at widely separate points and form a miniature meteorological network; they each measure pressure, temperature, acceleration, and cloudiness and are accurately tracked from Earth to give wind profiles. The bus, in entering the upper atmosphere, measures the neutral and positive-ion composition to supplement the data from the Orbiter. One of the probes is considerably larger than the other three and carries additional instruments to measure composition (mass spectrometer and gas chromatograph), radiation fluxes, and cloud particle properties. Though there are many particular objectives, a general theme is the meteorology and energy balance of the atmosphere below the clouds: how the atmosphere moves and the surface stays hot.

The Orbiter carries 12 experiments, two of which study the meteorology and energy balance in visible and thermal infrared radiation. The local, detailed views from the probes are thus taken to the global scale. There are 5 aeronomy instruments that measure the upper atmosphere and ionosphere directly during the lowest part of each orbit, which can be as



Ultraviolet picture of Venus from Mariner 10, 450,000 miles up.

close as 150 km to the surface. Additional information comes from radio occultation measurements and the deceleration of the spacecraft due to the atmospheric drag. Three more instruments measure the fields and plasma streaming by from the Sun. All 8 taken together should tell a great deal about the interaction of the two media. The radar will give a low-resolution reflectivity map of one hemisphere along with altimetry of the track below the spacecraft. Information on the interior can be obtained from gravity data and any internal magnetic field that may turn up. Finally, the Orbiter carries a gamma-ray burst detector; the long Earth-Venus baseline gives excellent direction-finding abilities for these mysterious events. The Orbiter will make one revolution per day for a full Venus year (243 days), and could continue even longer if it is still working.

Though Pioneer Venus is a NASA mission, managed by its Ames Research Center and built by the Hughes Aircraft Company, two of the aeronomy instruments are supplied by the Federal Republic of Germany, and a French scientist participates in the cloud-measuring experiments.

Over the years during which the Pioneer Venus hardware has been



Pioneer's Multiprobe: test model and artist's impression (right).

planned and built, much has been learned about the planet, but there are still plenty of questions. The Soviet Veneras 5 and 6 (1969) extended the chemical analysis of the atmosphere that had begun two years earlier with Venera 4 and Mariner 5. Venera 7 (1970) finally reached the surface and confirmed the temperature and radius deduced less directly by radio and radar. Venera 8 (1972) showed that some sunlight penetrates to the surface, and measured the natural radioactivity of the rocks. Mariner 10, on its way by to Mercury, took the famous photographs of cloud structures (left), confirming the 100 m/s winds, and made measurements of the upper atmosphere. Veneras 9 and 10 (1975) deployed probes and orbiters. Each probe took a panoramic picture of the nearby surface and made other measurements of surface properties.

On the way down, the probes showed that the clouds extend from 65 down to 40 km, and confirmed the Earth-based deductions that the principal particles are H_2SO_4 , about 1 μm radius. A mass spectrum of the atmosphere was also obtained. Orbiter data recently published suggest that the upper boundary of the ionosphere has been traced from a low height (around 270 km) in the subsolar region to a much higher value (over 600 km) near the terminator. It is possible that similar missions may be launched in August at about the same time as the Pioneer Venus Microprobe.

It may seem strange that the Viking of two years ago is not being followed by further exploration of Mars. Much of the explanation lies in the very long times that are needed to plan and execute a planetary mission. A follow-on to Viking would have had to be started at least six years ago, when construction of Viking had barely started. Even now, we have not fully assimilated the Viking results, and the form of the next mission is not clear. The activities that led to Pioneer Venus started in 1966, before the encounters of Venera 4 and Mariner 5. Getting it into the NASA budget and through the Congress took from 1970 to 1974, and hardware activities have been going on ever since. Planetary explorers must take the long view. The United States will return to Mars some day, and even to the Moon and Mercury. In the meantime, the Pioneers are going to Venus, and an earlier one will soon encounter Saturn; the Voyagers are on their way to Jupiter, Saturn, and perhaps Uranus; and Galileo is starting its long hardware phase, for a launch to Jupiter in 1982. The waits are long, but the excitement of exploration makes it worthwhile in the end. □

Out of the frying pan

THE English call it mince-meat; the Americans ground beef. But, made into patties, and grilled, barbecued or fried, it is known worldwide as hamburger. It was named for the great Hanse town at the mouth of the Elbe. Unfortunately, there is no city named Cheeseburg, and, although a cheeseburger contains cheese, a hamburger certainly does not contain ham. Possibly the most misbegotten descendant of the hamburger is the nutburger, a culinary and etymological horror that was spawned, of course, in southern California.

When animal or vegetable materials are charred or pyrolysed, carcinogenic derivatives of polycyclic hydrocarbons are produced. Our pithecanthrope ancestors must have dosed themselves with these chemicals as, crouched in caves, they breathed wood smoke and gnawed on burned meat. Their descendants gather around barbecue fires to inhale cigarette smoke while they watch the fat drip from steaks and chops onto red-hot coals from which the smoky flames arise to deposit benzopyrenes on the victuals. The carcinogenic effect of the benzopyrenes may be enhanced by alcohol in beverages that are quaffed in anticipation of the feast.

Last year, Sugimura reported that mutagens were produced when fresh whole fish and beef meat were broiled on steel mesh over a gas flame or charcoal. The smoke condensates from similarly-treated L-tryptophan yielded gamma-carboline derivatives that were very potent mutagens. This may mean that tryptophan in meat is the origin of some of the mutagens formed by pyrolysis. The key word "hamburger" was not used in describing the results, and the attention of the newspapers was apparently not aroused. But the fat was in the fire when a group at Washington University, St. Louis, announced, with an accompanying press release, that well-done pan-fried hamburgers contained mutagens that were not present in uncooked, lean, ground beef. The authors recommended cooking "in a microwave oven or under the element of an electrically-heated broiling oven" to avoid formation of mutagens. From the public response to this news it seemed that hamburgers are more famous and have more friends than even apple pie. A newspaper columnist, William Raspberry, said: "When danger waits at every hand, the warnings no longer lead us to safety. They lead us only to anxiety."



THOMAS H. JUKES

... When nearly everything is reported as dangerous, we are forced to behave as though nothing is dangerous".

Carol Tucker Foreman, US Food and Consumer Services Secretary, is well-known for her efforts to protect consumers from hazards of nitrates in bacon. I wonder how this risk compares with leaving hamburgers in the frying pan for an extra minute. I also wonder how to equate the danger caused by less than two nanograms of the estrogen diethylstilbestrol in a meat patty against the effects of what happens when the same meat receives a baptism of fire. Oncologists often declare that no-one knows how much or how little of a carcinogen will produce cancer. Raspberry said: "It would help us if scientists would tell us which substances are more dangerous than others, or in what levels these dangerous substances can be treated as benign. But they won't tell us these things; they say they don't know". It may be time to try and make some educated guesses. Incidentally, Sugimura noted a finding by Mizusaki *et al.* that the mutagenic activity of the tar derived from tobacco is proportional to the protein content of the tobacco leaves.

We recently were told that fat in the meat we eat increases the risk of cancer of the colon, and that hamburgers should therefore be thoroughly cooked so as to remove most of the fat. Unless the cooking process is carefully controlled, the choice between rare and well-done meat seems a dismal one. We await the next news from the carcinologists.

correspondence

Difficulties in assessing carcinogenic activity

SIR.—Extensive research projects to examine the validity of short-term tests for carcinogens are being carried out. The distinction between carcinogens and inactive compounds is often not clear. Consideration of the data on some of the pairs of active and inactive compounds illustrates this. The difficulties include the limitation of the number of animals that can be used and frequently the dose of material which the animals can tolerate. In comparing pairs of related compounds the product assigned as inactive should have been tested at higher levels, say 10 times, than the related carcinogen.

The process or processes of carcinogenesis are complex and probably involve several stages. In addition to complete carcinogens and "activating enzyme systems", initiators, promoters, cocarcinogens, antioxidants and other factors are involved. The assessment of carcinogenic activity itself is difficult; in many cases it can only be stated that the available data are such that "no evaluation of the carcinogenicity of this compound can be made". Even among the substances which are accepted as carcinogens there is a millionfold difference in potency as expressed in the dose of saccharin compared with aflatoxin needed to induce tumours. There are compounds such as phenobarbitone and DDT which are considered to be carcinogenic by some criteria but are not universally accepted as such. The proof of the negative, that a substance is not carcinogenic, is even more difficult and in many cases the tests so called "noncarcinogens" are inadequate.

2-Naphthylamine is carcinogenic to dogs, monkeys, hamsters and mice in large doses but not carcinogenic to rats. Although 1-Naphthylamine appears to be noncarcinogenic in hamsters and possibly in dogs, it has induced tumours when given to newborn or adult mice.

3,4-Benzopyrene is one of the most thoroughly investigated carcinogenic compounds and is a complete carcinogen. Pyrene is, however, a potent cocarcinogen (Hoffmann & Wynder *J. Air Pollut. Control Ass.* 13, 322; 1963 and Van Duuren & Goldschmidt *J. Nat. Cancer Inst.* 56, 1237; 1976) and in the complex process of cancer induction it is probable that cocarcinogens are important.

β -Propiolactone is carcinogenic on injection to rats and mice or skin application to mice. Although γ -Butyrolactone was not active by oral administration or injection in mice (Rudali, Boyland & Castegnaro *C.R. Acad. Sci.* 282, 799; 1976) it appeared to induce cancer in rats by oral administration (Schoental *Israel J. Med. Sci.* 4, 1146; 1968).

9, 10-Dimethylanthracene is a weak carcinogen on application to the skin, but inactive on injection in mice. Although anthracene was not carcinogenic when tested by skin application to mice, it

induced sarcomas on injection in rats; (Schmähl *Zeit. Krebsforsch.* 60, 697; 1955) naphthalene was inactive when tested at the same time.

2-Acetylaminofluorene is one of the widely used experimental carcinogens that might have been used as an insecticide in food had tests not shown it to be carcinogenic. 4-Acetylaminofluorene has not been adequately investigated to say that it is inactive and in one experiment it induced cancer of the colon in one of ten rats.

Urethane is a complete carcinogen; although *O*-isopropyl-N-3-chlorophenyl has not been shown to be a complete carcinogen, it acts as an initiator of tumours in mice skin when the original application is followed by treatment with croton oil.

With some types of compound chemical and biochemical considerations give indications of inactivity that may be more convincing than the available biological data. Thus the reactive 3 position of the reactive metabolite of 4-Nitroquinoline *N* oxide is blocked in 3-methyl-4-nitroquinoline *N* oxide, so that the latter is inactive. Similarly, in 3,3',5,5'-tetramethylbenzidine the reactive *ortho* positions to the reactive hydroxylamine metabolite are blocked with methyl groups and the compound is inactive.

These examples show how difficult it is to be sure that compounds can be free of cancer hazard from animal tests. It is difficult to show that toxic compounds such as arsenic and selenium derivatives are carcinogenic to animals because only small doses can be given. On the other hand, substances with low acute toxicity such as saccharin and trichlorethylene have produced tumours because they can and were given in large amounts. The significance of the results of tests in which large amounts of otherwise harmless substances are administered is difficult to evaluate.

The uncertainty of negative results and the great quantitative variation in carcinogenic activity must be considered when short-term tests are compared with conventional tests for carcinogenic activity.

Yours faithfully,

ERIC BOYLAND

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Was war the origin of intelligence?

SIR.—In the review by Glynn Ll. Isaac on the development of early man (*Nature* 273, 588; 1978) he suggests the question: "Was the enlargement of the brain in the Homo lineage(s) a response to the demands of culture?—or might a somewhat enlarged brain, at least at the outset, have been a preadaptation which facilitated the development of culture and technology?"

It is difficult to believe in preadaptations, and as I have suggested earlier (Fremlin, J. H. *Be Fruitful and Multiply* Rupert Hart-Davis 1972) the exceedingly rapid expansion of the brain is much more easily understood in terms of lethal competition between small closely-related groups. By the time our ancestors had developed the most elementary stone weapons they must have been very unattractive prey for the big carnivores and their subsequent population numbers must surely have been limited by direct competition between groups for food and the territory containing it. While a very moderate degree of intelligence is sufficient for the out-manoeuvring of the big cats, there is no limit to the level of intelligence useful in competing with one's own species; whatever the current general level of intelligence, there is a great military advantage to any group which has a higher level still.

Yours faithfully,

J. H. FREMLIN

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Support for Spanish scientists

SIR.—The article 'Spain has forgotten her scientists' (6 July, page 8), should undoubtedly bring the problems of Spanish science to the attention of important sectors of opinion in Spain and abroad.

We would like to emphasise the enthusiasm and dedication of a substantial number of Spanish scientists, who feel capable of giving Spanish science a strong push forward if the minimal financial aid and basic facilities are provided. This is in contrast to the traditional apathy the Spanish have towards research, and their peculiar lack of confidence in their own scientists. The lack of science in the programmes of the political parties (mentioned in the article) is just a reflection of the lack of interest in the population as a whole. This, in turn, shows clearly the poor educational standards of the country.

Industry entirely owned by Spanish capital is very rare, and does not promote research; multinationals prefer to do their research in other, scientifically developed, countries. However, in both cases, this situation will change when the average quality of Spanish research becomes respectable. It is not yet appropriate to discuss the distinction between pure and applied science: applied research will become possible only after a body of 'pure scientists' has been developed from which future 'applied' scientists will be trained, recycled, and with whom at all times, they will be able to interact.

Yours faithfully,

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news and views

Pluto's satellite

from David W. Hughes

PLUTO the lone outsider of the Solar System is lonely no more. The planet has a satellite. This new discovery was made by J. W. Christy of the United States Naval Observatory, the same observatory that was responsible for the discovery of Phobos and Deimos, the moons of Mars. The discovery was announced in *Circular 3241* of the *International Astronomical Union's Central Bureau for Astronomical Telegrams* published on 7 July. Christy had noticed elongations of the photographic image of Pluto on plates taken with the Naval Observatory's 155-cm diameter astrometric reflector on 13 and 20 April and 12 May of this year. Checking back through previous photographic plates revealed similar elongations on 13, 15, 16, 17 and 19 June in 1970 and on 29 April and 1 May, 1965. The maximum elongation was 0.9 s arc. Analysis of the position angles of the elongation indicates that the satellite is orbiting the planet with a period of 6.3867 days. This is the same as the spin period of Pluto (see Anderson & Fix, *Icarus* **20**, 279; 1973).

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So the two motions have become locked to each other, Pluto's satellite always remaining above the same point on the planet's surface.

The data suggests that the mean distance between Pluto and the satellite is 20,000 km. The mass of Pluto M_P can now be estimated by substituting

$$M_P = 4\pi^2 d^3 / GT^2$$

where d is taken to be the mean satellite-planet distance, T is the satellite's orbital period, G is Newton's constant of gravitation and the satellite is assumed to be much less massive than the planet. Pluto's mass turns out to be 1.56×10^{25} g, a value which lies beautifully within the uncertainty limits of the values given by Cruikshank *et al.* (*Science* **194**, 835; 1976; see *News and Views* **266**, 307; 1977). Pluto is 1/380 the mass of Earth. Cruikshank *et al.* concluded that the composition of Pluto is probably dominated by frozen volatiles, like most of the outer planetary satellites, and thus has a density around 1.5 g cm^{-3} . Combining this value with that obtained above for the mass gives Pluto a diameter of 2,700 km. The

surface albedo is about 0.6.

The data obtained by Christy also suggest that the satellite is 2 to 3 magnitudes fainter than Pluto (that is about 10 times less bright). Assuming that the satellite has a similar albedo to Pluto's and that reflected brightness is proportional to the surface area exposed to the radiation gives the satellite a diameter of around 850 km.

The satellite orbit is nearly circular and is inclined at about 105° to the plane of the sky.

The existence of the satellite was confirmed on exposures made with the same telescope on 2 and 5 July and on an exposure made by J. A. Graham using the 400-cm reflector at the Cerro Tololo Interamerican Observatory on 6 July. *IAU Circ. 3241* gives a brief ephemeris and stresses that further observations are desirable.

The satellite has been given the rather unglamorous designation of 1978 P.1, and the world will have to wait for a meeting of an IAU nomenclature committee before it is given a proper name. Might I hazard a suggestion. How about Persephone, the fair goddess whom Pluto set on his throne in Hades and crowned as his queen. □

Influenza A viruses: shaking out our shibboleths

from Francis A. Ennis

THE studies of Nakajima, Desselberger and Palese (this issue of *Nature*, page 334) on the comparison of the recently isolated 'Russian' strains of influenza virus with earlier members of the H1N1 subtype, which circulated between 1946 and 1957, present a challenge to many of our existing ideas on the behaviour of influenza A viruses. Their results confirm earlier findings of close similarity between the surface haemagglutinin (H) and neuraminidase (N) antigens of the Russian strains and strains isolated in 1950 (*WHO Weekly*

Epidemiological Record **53**, No. 2, 16; 1978). Palese and his colleagues have now extended the comparison beyond the limits imposed by serological tests by comparing oligonucleotide maps of the genomic RNAs of the virus strains. On the basis of these maps they postulate that the current Russian strains are identical with the viruses which circulated in 1950.

Is this coincidence, or has the 1950 virus re-emerged in its original state? The authors suggest that the latter alternative is more likely and offer three possible explanations of the persistence of the genetic information unchanged: persistence in man without mutation; passage in an animal

reservoir without rapid genetic change; or the possibility that the virus was truly frozen and only recently reintroduced to man. The first two possibilities have been considered by many virologists, but lack supporting evidence, while Chinese and Soviet scientists have denied the third possibility as the origin of the recent epidemics in their countries. I will not attempt to make a premature decision on the origin of the recent strains, which would probably be proved as incorrect as some of our other beliefs regarding the behaviour of influenza A.

Together with other recent experience, the re-emergence of H1N1 has shattered some solidly held conceptions.

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It was generally believed that a localised outbreak of influenza would be due to a single antigenic variant which would displace previous variants. Last winter there were many examples throughout the world of outbreaks caused by two antigenically distinguishable viruses of the H3N2 subtype, that is, A/Victoria and A/Texas which caused disease in individuals of all ages. In addition the H1N1 'Russian' strain caused outbreaks of illness confined mainly to people less than 25 years old while the A/Victoria and A/Texas variants were concurrently causing disease in older individuals (Kendal *et al.* *Am. J. Epidemiol.* in the press).

Another casualty is the concept that a subtype would not recycle until many decades later, when few of the population would be immune as a result of previous exposure. The epidemics caused by the recent H1N1 strains, reported by the Russians in late 1977, and later confirmed by the Chinese as having occurred as early as May 1977, have broken out only two decades after the replacement of that subtype by the Asian (H2N2) subtype in 1957. The recent H1N1 virus strains have spread successfully and caused outbreaks around the world despite the fact that individuals over 25 years of age were immune as a result of their earlier exposure.

Another supposedly fixed characteristic of influenza viruses is their virulence for man. With the possible exception of the virus causing the high mortality of the 1918 pandemic, influenza A viruses seem to have a relatively constant virulence, which was sufficient to produce detectable excess mortality even in regional epidemics. This was not the case with the recent H1N1 strains of virus which apparently caused no excess mortality, as well as very little illness in people over age 25 years. The virus was transmitted very effectively in young non-immune subjects but not to individuals who were older. It might be argued that the absence of illness, and associated deaths, in older individuals was due to their solid immunity, and not to the lack of virulence of these recent strain; however, influenza A viruses have repeatedly been shown to cause illness and death in populations with considerable previous exposure to antigenically similar viruses of the same subtype.

Thus the behaviour of influenza A viruses seems to have become more complex. However, some of these changes may be related to changes in our ability to detect and monitor epidemics rather than to changes in the virus. At least one of the above events may be explained by the more intensive surveillance of influenza A virus

activity as a result of WHO support. It may be that past outbreaks were caused by cocirculation of antigenically distinct members of a given subtype which were not detected. Better surveillance and more specific antisera may be the reason for the isolation of the A/Victoria and A/Texas variants of the H3N2 subtype in outbreaks last year. It is not likely, however, that widespread outbreaks of influenza due to the viruses of the H1N1 subtype have gone undetected since these

viruses were 'replaced' by the Asian virus of the H2N2 subtype in 1957. The surface antigens of the H1N1 strains are readily distinguishable from those of the other influenza A subtypes by simple serological testing.

The detailed characterisation of the genome of influenza A viruses, should provide a sounder basis for future estimates of the source, and expected behaviour, including virulence in man, of strains of these viruses. □

The origins of modern biochemistry

from Robert Olby

A RECENT symposium on the origins of modern biochemistry* represented an attempt to bring scientists and historians together to review the history of a discipline. It is recognised that in oral history by private interview the scientist may aggrandise his or her contribution; and such claims may go unchallenged. In the more public environment of a small invited audience consisting of scientists with rival claims, each with their own perception of the history of the discipline, and with historians familiar with the subject, it was hoped that a more objective reconstruction might be possible.

There is no doubt that much valuable information will be made available when the invited papers and discussions are published. F. Holmes's (University of Western Ontario) unravelling of the early theories of protein metabolism showed the remarkable longevity of the concept of 'direct' assimilation, that is, the ingestion of proteins in the form in which they are assimilated, and the extent of the conceptual shift to the theory of assimilation from the constituent amino acids as in the *Baustein* Theory of Kossel. Sarah Ratner (US Public Health Office) gave a very personal account of the first studies of amino acid turnover using ^{15}N and deuterium in Columbia University and brought home to us the difficulties faced by those who pioneered the tracer technique. It initiated a lively discussion of the extent to which the concept of dynamic metabolism depended upon or was independent of the use of radioactive isotopes.

On the historiographical side there was a welcome absence of the tendency to dismiss or ignore research which has

failed to find a place in contemporary knowledge. Thus rivals to the polypeptide theory of protein structure, and protein synthesis as the reversal of proteolysis were given due space. Several speakers covertly implied the need for revision of the popular view of the origins of molecular biology according to which the physicists' intellectual migration to biology under the spell of either Niels Bohr or Erwin Schrödinger played a dominant part. It is, indeed, remarkable that in contrast to this emphasis so little attention has been given to the role of Harold Urey who discovered deuterium, and devised methods for concentrating it and the naturally occurring isotopes ^{13}C , ^{15}N , ^{17}O , ^{18}O , ^{33}S and ^{34}S . The work of the Rockefeller Foundation in supporting research on phage and X-ray crystallography of the proteins is well known. The Foundation's parallel support for the application of isotopes in biochemistry—especially of Urey's colleague, David Rittenberg, in Schoenheimer's laboratory in Columbia University—deserves more prominence.

The resulting 'tracer' technique was used by Cohen to reveal the temporal relation between DNA synthesis and protein synthesis of phage, by Hershey and Chase to follow the fate of phage protein and nucleic acid in progeny phage, and by Hevesy to contrast the slow turnover of DNA compared with proteins. The fundamental concepts of rapid turnover and the existence of a metabolic pool were promoted and established by tracer experiments. They have been fundamental to our understanding of molecular processes in protein synthesis.

Discussions of the protein chemists' studies of plant viruses were suggestive of several features of what T. S. Kuhn has called 'normal science', that is,

*A New York Academy of Sciences Symposium; held on 31 May–2 June, 1978. The proceedings will be published by the New York Academy of Sciences.

puzzle solving within an organised structure of theoretical commitments and experimental techniques. Since tobacco mosaic virus is constituted of 95% protein and since protein was regarded as the material basis of biological specificity, Stanley looked upon the 5% phosphorus discovered by Bawden and Pirie as an impurity. When attempts to alter the protein by oxidation, methylation and decarboxylation failed to destroy infectivity or yield mutants, it was simply concluded that such chemical changes were not sufficient to alter the biological specificity of the virus particle. This view was taken despite the fact that treatments specific for nucleic acids—deamination with nitrous acid and irradiation with ultraviolet light in the region 2,600 Å both inactivated the virus. Moreover, viral protein on its own failed to show infectivity. These 'anomalies' in the virus research programme of the protein chemists were accommodated by the introduction of accessory hypotheses—the energy transfer hypothesis for ultraviolet light and the integrity of the entire nucleoprotein virus particle as essential to infectivity. H. Frankel-Conrat, in his masterly survey of the early work on the chemistry of plant viruses, responded to Seymour Cohen's question: why did those in virus laboratories where relatively undegraded viral RNA had been isolated in the 1940s not test the RNA for infectivity at the time? They had been impressed by the fact that nearly all the virus particle was made of protein, and that the amino acid composition of this protein showed differences in different taxonomic forms. No parallel evidence of nucleotide differences was evident at that time in the nucleic acid portion of the particle. There was no positive motive to test the infectivity of viral nucleic acid. Whether the protein had shown infectivity or not however, it would surely have been standard procedure to test the nucleic acid as a control, an action all the more to be expected when the protein fragment proved negative. Failure to test, even as a control, the infectivity of the viral nucleic acid may be seen as an expression of commitment to the paradigm of the protein theory of specificity.

In the round-table discussion and in questions put to the contributors, there was evidence of different emphases and aims. Scientists' questions were largely, though not entirely, devoted to the clarification of the course of intellectual and experimental developments in the science, whereas historians were asking questions about the extent of

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Monkeys prefer kin

from John Krebs

It is perhaps not surprising that in many higher vertebrates parents and young can recognise each other as individuals. In a busy, crowded colony of guillemots (*Uria aalge*) newborn chicks respond by approaching only to the calls of their own parents, ignoring the hundreds of other similar calls in the close vicinity. The parents too respond to the calls of their own young but ignore other young in the colony (Tschanz *Z. Tierpsychol. Beiheft* 4, 1; 1968). In most cases, this sort of individual recognition comes about through learning: young guillemots learn their parents' calls while in the egg, and to quote another example, female goats learn the individually characteristic scent of their own offspring during the first few minutes after giving birth (Klopfer & Klopfer 1966. *Z. Tierpsychol.* 23, 588; 1966).

A much more startling (or "incredulous" as one delegate put it) result, was reported by H. Wu and G. Sackett (University of Washington) at the 1978 annual conference of the (American) Animal Behaviour Society*. They described an experiment showing that young rhesus monkeys (*Macaca nemestrina*) can recognise their half-brothers or half-sisters without any previous contact with them. The 16 test monkeys were related to their half-siblings only through their fathers, thus neatly eliminating the possibility that some family-specific cue such as odour might have been acquired *in utero* from the mother. The young monkeys were reared in strictly controlled, semi-isolated conditions and given just enough daily contact with unrelated peers to ensure normal social development. They were tested once at ages ranging from 44 to 344 days in a choice apparatus in which they were offered the chance to face towards, and later to enter, three compartments containing respectively a caged half-sib, a caged non-relative and an empty cage. The test monkeys had never had any previous contact with any of the stimulus monkeys.

contact between laboratory groups and disciplines, the causes of discipline insularity and the means of communication. To the scientists the value of history of science in teaching was seen largely in its power to clarify the nature of scientific concepts. Young aspiring scientists often expressed little interest in the history of their subject. This attitude tended to change once

Thirteen of the sixteen spent more time in the half-sib compartment than in the non-kin section, while eleven out of the sixteen showed a similar preference in the initial part of the experiment where they simply faced one or other compartment. The results were not influenced by age or sex of the test monkeys. The remarkable conclusion is that rhesus monkeys have an inborn ability to recognise half-siblings, and presumably other close relatives. At the same conference, W. Holmes (University of Washington) showed that a similar sort of kin recognition may occur in Arctic ground squirrels (*Spermophilus parryi*). Holmes's results were based on cross-fostering siblings starting at the age of 3 to 7 days, so although they demonstrated kin recognition, they did not distinguish between an inborn discrimination and one based on early learning.

How does a monkey recognise its half-sib? The obvious possibility is by comparison with itself, an idea suggested some years ago in an experiment with day-old chicks by E. Salzen and J. M. Cornell (*Behaviour*, 30, 44; 1968). They dyed isolated chicks different colours, and found that they subsequently tended to prefer to join groups with feathers dyed the same colour as their own. Wu and Sackett are now in the process of trying to find out if rhesus monkeys have similar but more sophisticated narcissistic tendencies as young chicks.

The theory that social cooperation evolved through kin selection has been remarkably successful in predicting details of social behaviour in animals ranging from bees to lions. Wu's and Sackett's results suggest that the possibilities of kin recognition, and therefore of kin-directed cooperative behaviour which might be favoured by selection, are far greater than previously supposed.

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*Held at the University of Washington, Seattle, on 19–23 June, 1978.

they became established in the profession and had made a contribution to the discipline. Historians wanted to emphasise the wider educative role of their subject as a contribution to an education in our cultural heritage. As such it is concerned not only with 'internal' conceptual and technical developments but with the interface between science and society—the reasons for

funding science, the paths to application and exploitation—and with the professionalisation of science and discipline differentiation within the sciences.

The history of science has come a long way from mere 'signpost' history, from Whiggish interpretations of past science which have served to justify the orthodoxy of the present, and from an insularity to the social and political context of the sciences. The initiative of the New York Academy of Sciences in bringing scientists together and including some historians is to be welcomed, not only as a means of adding to the sources for the history of science but in promoting personal contact between scientists and historians. □

Malaria, piroplasmosis and endotoxin

from F. E. G. Cox

ONE of the most unexpected observations to emerge from the massive amount of work directed towards an understanding of immunity to intraerythrocytic protozoa is that previous exposure to BCG or *Corynebacterium parvum* protects certain strains of mice completely against *Babesia* spp. and partially against *Plasmodium* spp. (see Cox *Nature* **273**, 623; 1978). BCG and *C. parvum* also sensitise mice to endotoxin and elicit a protective response against certain tumours (Snider *et al. J. natn. Cancer Inst.* **60**, 785; 1978, Milas & Scott *Adv. Cancer Res.* **26**, 257; 1978). The implication has been that these agents or their products in some way cause macrophages, after stimulation by endotoxin, to release a soluble product that is instrumental in the destruction of rapidly dividing tumour cells (Carswell *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 3666; 1975). The release of such a substance is also brought about by the yeast cell wall polysaccharide, zymosan, and the discovery that zymosan also protects mice against *Babesia* spp. and *Plasmodium* spp. has led I. A. Clark of Canberra to put forward a new hypothesis linking sensitisation to endotoxin with the intraerythrocytic death of blood parasites and the pathogenesis of malaria and piroplasmosis (*Lancet* **ii**, 75; 1978).

Clark has shown that during the course of infections with *Babesia microti* in CBA mice the animals become

increasingly sensitive to endotoxin in the form of bacterial lipopolysaccharide (LPS) being several hundred times more sensitive at the peak of the infection than at the beginning or end. Similar results were obtained with *B. rodhaini* and the malaria parasites *Plasmodium vinkei petteri* and *P. berghei*. It seems reasonable, therefore, to assume that mice become sensitised to endotoxin during the course of the infection and at the peak, when presumably vast amounts of parasite material are being released, a phenomenon analogous to endotoxin challenge occurs. This causes macrophages to release soluble factors that kill the parasites within the red cells resulting in the crisis associated with resolving infections. The intracellular death that occurs at the crisis is seen both in naturally resolving infections and in mice pretreated with BCG or *C. parvum* and challenged with large numbers of parasites.

Endotoxin shock has been shown to produce many effects including polyclonal B lymphocyte stimulation and depression of T lymphocyte activity both of which are associated with the crisis in malaria infections. The pathology of malaria and piroplasmosis is similar and in malaria in particular it has long been thought to have much in common with endotoxin shock (Neva *New Engl. J. Med.* **277**, 1241; 1967). Thus all the events that occur during an infection could well be part of the same process.

If the death of the parasites is brought about as a result of endotoxin shock it might be expected that anything that sensitised mice to endotoxin would have the same effect and Clark has produced evidence to support this by showing that *Brucella* and *Coxiella* protect mice against intraerythrocytic protozoa as effectively as BCG and *C. parvum*.

This new hypothesis poses as many questions as it answers. It is not clear, for example, whether or not the parasites themselves produce endotoxin, for this has certainly not been easy to detect, but this may be because the quantities involved are so small. Alternatively, sensitisation may occur through the gut flora, for the permeability of the gut is known to be affected by blood parasites, or by antigen-antibody complexes (a theory that would be attractive to immunologists). The nature of the actual macrophage product is still obscure. It might be tumour necrosis factor (Carswell *et al. Proc. natn. Acad. Sci.* **72**, 3666; 1975) or it could even be arginase, which has recently been shown to be produced by zymosan and endotoxin-stimulated macrophages (Currie *Nature* **273**, 758; 1978). Arginase brings about arginine

deprivation which presumably could adversely affect the rapidly dividing intraerythrocytic parasites. The problem is that we simply do not know enough about the biochemistry of either the parasites or the various macrophage products that might affect them.

The big question, however, is how relevant this is to human and veterinary medicine. Here Clark produces a mass of circumstantial evidence to suggest that his hypothesis is a general one. It remains to be seen whether or not the weight of contradictory evidence which will inevitably be produced outweighs this supporting evidence. There are some exciting times ahead in this new area which is one with which few parasite immunologists have so far become involved. □

Hormone binding in plants

from C. J. Lamb

THE existence of plant growth regulators such as auxins and cytokinins has been known for some time and a plethora of physiological responses to each class of hormone have been described. However, little is known of the biochemical basis of plant hormone action. By analogy with animal hormones one might expect receptor proteins which bind the hormone and as a result of this interaction, initiate the biochemical reactions leading to the physiological response. Characterisation of such receptor proteins is of considerable interest since they are potential sites for the action of herbicides and synthetic growth regulators, and recently a number of workers have overcome considerable theoretical and technical difficulties to characterise hormone binding sites with the properties expected of plant hormone receptors.

There are no known receptor mutants in plants and the primary responses to hormones have not yet been elucidated. Therefore indirect methods are needed to assess the biological relevance of plant hormone binding. An excellent illustration of the attendant problems is provided by the work of Sussman and Kende¹ on cytokinin binding to a particulate fraction from cultured tobacco cells. Bound hormone can be separated from free hormone by sedimentation of the par-

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ticulate fraction and using this technique with high specific activity [^3H] N⁶-benzyladenine (BA) revealed at least two types of saturable cytokinin binding sites against a high background of unsaturable binding. The major saturable component had a low affinity for BA ($K_d = 7 \times 10^{-6} \text{ M}$) and was resistant to heating. The second site occurred at much lower frequency, could not be detected with radioactive hormone of low specific activity, was heat labile and had a high affinity ($K_d \sim 10^{-7} \text{ M}$) for BA. Furthermore, the ability of a series of halogenated analogues of BA to bind to the high affinity site correlated well with the cytokinin activity of these analogues in a number of bioassays including stimulation of cell growth and division in tissue cultures and induction of bud formation. In contrast, there was no correlation between biological activity and binding to the low affinity site. This site shows some of the features of talcum powder, a non-biological material which binds cytokinins in a saturable but non-specific manner. The high background of biologically irrelevant binding together with the lack, until recently, of high specific activity radioactive cytokinins have hampered the search for cytokinin receptor sites, but the high affinity site reported by Sussman and Kende seems to have the required properties.

In contrast, the search for auxin receptors in extracts of maize coleoptiles has borne considerable fruit. Physiological effects of auxins include stimulation of the extension growth of plant cells, induction of vascular differentiation and inhibition of lateral bud development. An important component of auxin action is their preferential movement from the apex towards the base of plant stems. This polar transport is thought to be involved in the regulation of expansion growth of cells below the tip, and in the inhibition of lateral bud development. An initial report of saturable binding of the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) to a particulate fraction in maize coleoptile extracts² has been followed by demonstration of particulate binding sites for the auxins 3-indolylacetic acid (IAA) and 1-naphthylacetic acid (NAA)³. NPA did not compete for the auxin binding sites and *vice versa*. Further study of the NAA binding indicated that a major proportion, but not all of the binding sites were associated with the endoplasmic reticulum (ER)⁴ and that the affinity of a variety of auxin-like molecules for the site correlated reasonably well with auxin activity in a bioassay based on promotion of cell expansion growth⁵. A non-dialysable, heat-stable supernatant factor was found which narrowed the specificity spectrum without

The polyp has no I's

from Jonathan Slack

EVER since Abraham Trembley's work in the eighteenth century, the regenerative powers of *Hydra* have made it a popular organism among experimental biologists. Controversy has raged over the relationship of the various cell types to each other; in particular over the role of the interstitial cells (I cells) which are interspersed between the epitheliomuscular cells of the outer layer and the digestive and gland cells of the inner layer.

Recently some clear results which should dispel the controversy have been obtained by Beverly Marcum and Richard Campbell from the University of California at Irvine (see *J. Cell Sci.* **29**, 17; 1978; *Science* **197**, 771; 1977). Two years ago Campbell succeeded in making *Hydra* which were completely free of I cells by treatment with colchicine (*J. Cell Sci.* **21**, 1; 1976). These animals are rather feeble and have to be fed by hand, but it has proved possible to propagate stocks asexually and some lines have remained completely free of I cells. They also lack nerve cells and nematocytes but have their normal complement of epitheliomuscular and digestive cells, implying that these two tissues are mitotic and need not be continually restocked from the I cells.

Now Campbell and his colleagues have shown that the principal morphogenetic properties of the I cell-free animals are quite normal. When they are cut in two, both halves will regenerate with conservation of polarity. When a head is grafted into a gastric region it will induce a secondary axis from the host tissues. When a segment of the body is inverted between a head and a foot, it becomes repolarised by its surroundings at the same rate as in a normal animal.

The implications of these results are as follows. First, it is now clear

that the differentiated cell layers are capable of regeneration and that the positional information system which controls polarity can reside in these tissues. Second, differentiated nerve cells are not needed for regeneration, in spite of a widely held belief, based partly on data from a variety of other animals such as earthworms, starfish and amphibia, and partly on evidence such as the inhibition of regeneration by neuropharmacological inhibitors.

Third, they seem to cast doubt on one of the most popular candidates for a morphogen, in the form of a peptide isolated from neurosecretory granules and which increases the tentacle number of regenerating animals (see *J. Embryol. exp. Morph.* **29**, 27; 39; 1973; *Cell Diffn.* **5**, 13; 1975). This factor was thought perhaps to be a component in the mechanism proposed by Meinhardt and Gierer (*J. Cell Sci.* **15**, 321; 1974) which has been applied to a number of embryological problems and attracted some support, but it seems that the significance of the head activating substance will now have to be reassessed.

In the same issue of the *Journal of Cell Science* as Campbell's paper, T. Sugiyana and T. Fugisawa report the isolation of an I-cell free mutant which has properties similar to those of the stocks prepared by colchicine treatment (*J. Cell Sci.* **29**, 35; 1978). They show that I cells will migrate into such a creature from a normal graft, so that it is possible to create chimaeras in which the main tissue layers come from one strain and the I cells and their derivatives from another. This should make a number of questions on cell interactions accessible to experiment.

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altering the number of binding sites⁶.

Meanwhile a second group has provided evidence for two binding sites with different affinities for NAA that could be partially resolved by sucrose density gradient centrifugation⁷. In a major advance these sites were solubilised by acetone treatment and purified 100-fold⁸. Binding of NAA to solubilised sites gave K_d values of $1.7 \times 10^{-7} \text{ M}$ and $1.4 \times 10^{-6} \text{ M}$: in the concentration range for half-saturation of auxin transport and physiological responses. The sites are probably proteins and gel exclusion chromatography gave

an asymmetric profile consistent with two incompletely resolved species of molecular weights 47,300 and 40,300. These values may be underestimates since in the April issue of *Plant Physiology*, Cross *et al.*⁹ report the solubilisation of auxin binding sites by Triton X-100 treatment of particles prepared in the presence of phenylmethylsulphonylfluoride (PMSF) to give detergent-protein micelles, molecular weight about 90,000. In the absence of the protease inhibitor PMSF, secondary peaks of auxin binding activity with much lower molecular weight were

obtained suggesting that the binding sites become susceptible to limited proteolysis following tissue homogenisation, a phenomenon previously observed during the purification of the photoreceptor phytochrome.

Recently Dohrmann *et al.* have characterised three classes of NAA binding sites¹⁰. In addition to the major site at the ER, a second site was distinguished by its low affinity towards phenylacetic acid. In sucrose density gradient centrifugation this site cofractionated with particle bound acid phosphatase, considered by these authors to be a marker for the tonoplast. The binding spectrum of this second site was unaffected by the supernatant factor but was quantitatively similar to the modified spectrum of the ER site in the presence of the factor and it was proposed that the two sites might be biogenically related. A third site was distinguished by preferential binding of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D). This site was on denser membranes than the first two sites and cofractionated with NPA binding activity and a glucan synthetase active only at high UDPG concentrations. Localisation at the plasma membrane was suggested. There is considerable physiological evidence that auxin transport occurs at the plasma-membrane and detailed studies of the kinetics of IAA influx and efflux with crown gall cells of virginia creeper grown in cell suspension culture have shown a saturable carrier for IAA¹¹. Interestingly, the carrier-mediated uptake of IAA is inhibited by 2,4-D, which is itself transported by the carrier, and it might be speculated that the third auxin binding site of Dohrmann *et al.* is involved in auxin transport. However, a functional relationship between the 2,4-D binding site and the binding site for the transport inhibitor NPA has not been reported.

Consideration of the roles of the auxin binding sites localised on internal membranes has centred on a possible involvement of the binding site on the ER in auxin-stimulated H⁺ secretion. There is much evidence that the initial effect of auxin on cell elongation in shoots is by induction of H⁺ secretion into the cell wall space (see ref. 12 for example). It has been suggested that auxin binding to sites on the ER might promote H⁺ transport from the cytoplasm to the cisternal space of the ER (ref. 4). The absolute latency of 10–20 min in auxin action on cell elongation and acid secretion would then be explained by the time taken for transport of the acid-containing particles from the ER to the plasma membrane. However, solubilised binding sites can be separated by gel exclusion chromatography from basal and K⁺-stimulated ATPase

activity and there is no auxin stimulation of ATPase activity in intact membranes⁹. These results suggest the binding site is neither itself a proton pumping ATPase nor a separate entity that modulates the activity of such an ATPase, although it is possible that the relevant ATPase activity was not detectable under the specific assay conditions employed. Alternative proposals are that auxin induced H⁺ secretion results from some other transport process, for example an auxin : H⁺ symport or from operation of an electron transport chain.

Clearly the next major tasks are to establish the functions of the binding sites in hormone action and to elucidate the events between hormone binding and physiological response. One would hope that the convincing demonstration of plant hormone bind-

ing sites with properties predicted for receptor sites is the prelude to a rapid increase in our understanding of the biochemistry of plant hormone action.

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Origin of the diffuse cosmic gamma rays

from Arnold W. Wolfendale

As each window on the Universe is opened, our knowledge increases but our understanding follows more slowly. The gamma-ray window is the latest and the interpretation of the view is still the subject of much debate.

The gamma radiation arriving at the Earth is characterised by two components: one, confined very largely to the galactic plane and peaked in the general direction of the galactic centre and the other having an intensity which appears to be roughly the same in every direction. It is natural to associate the former with processes occurring in the Galaxy and indeed some of these gamma rays have been positively identified as having come from the direction of known galactic objects (pulsars, H II regions . . .). The latter—the so-called diffuse gamma ray background—is more difficult to identify, and this is the subject of discussion in the present note.

With the exception of one or two peaks towards known extragalactic objects (to be examined shortly) the diffuse background is, necessarily, of constant intensity everywhere, although it must be stressed that the accuracy of measurement is rather poor so far. There is, therefore, immediately the problem, confronted in other parts of the electromagnetic spectrum, such as the X-ray and radio bands, of how much is coming from our own Galaxy and how much is coming from beyond. There are some who believe that the so-called halo of the Galaxy is so big that its γ -ray emission (probably by

way of the Inverse Compton collisions of cosmic ray electrons with starlight and 2.7 K photons) is a large fraction of the measured diffuse gamma radiation. If this is the case then it is a rather tame affair. Arguments against this attitude are twofold: the halo would surely be expected to give a spatial distribution of intensity inconsistent with the measured distribution (we are not at the centre of a near spherical halo) and, most important, the diffuse gamma rays seem to have a peculiar 'shoulder' in their energy spectrum which would certainly not be expected.

The shoulder, which shows itself as an excess of gamma rays between about 1 and 5 MeV, has itself had a chequered career. The early satellite experiments, typified by the Apollo missions, claimed evidence for a considerable excess and this triggered off considerable theoretical analysis—and the inevitable exotic explanations. However, as time went on it was realised that the data had been under-corrected for gamma rays generated in the spacecraft by the interaction of cosmic ray protons. These unwanted background gamma rays—which are also a nuisance in balloon experiments, where they come from the residual atmosphere above the detector as well as in nearby equipment—have proved to be most difficult to eliminate, or allow for, but it does look as though the corrections have now converged and that the quoted spectrum can be believed. The spectrum has been neatly summarised very recently by Floyd Stecker (*Nature* **273**, 493; 1978) and inspection of his intensity versus

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energy plot shows that the shoulder is still there, the intensity at 3 MeV being a factor five higher than expected on the basis of a power law of constant exponent fitted at 0.2 MeV and 100 MeV.

Accepting that the bump is genuine, what then is the most likely explanation of the diffuse gamma ray flux? Some of the flux must come from galaxies like our own and the contribution can be estimated fairly well. Calculations by Strong *et al.* (*J. Phys.* **A9**, 1553; 1976) show that only about 5% (at 3 MeV) can be explained in this way and one is immediately obliged to postulate more interesting sources (the same situation arises in X-ray astronomy) for the remainder.

No doubt the most exciting possibility is that cosmological effects are at work. This is an attractive idea because gamma rays have great penetration and it is natural to identify them as some sort of cosmological probe. The shoulder immediately suggests the existence of a smooth continuum with a broad peak (at a few MeV) superimposed and the peak can be identified with gamma rays from neutral pions produced in nucleon-antinucleon annihilation. Such a peak would appear at 67 MeV ($= M(\pi^0) c^2/2$) with respect to the pion's rest frame but would be seen redshifted to a few MeV if it had occurred at a suitable early stage in the evolution of the Universe. This idea has been pressed very vigorously by Floyd Stecker and associates and in fact Stecker regards the cosmological explanation of the diffuse gamma rays as providing considerable support for the baryon symmetric big bang cosmology. Clearly, the stakes are so high that one must examine the explanation for the gamma rays very carefully.

Strong *et al.* considered 'unusual' galaxies as well as 'normal' galaxies in their attempt to explain the diffuse gamma rays in terms of unresolved discrete sources. Radio galaxies were considered as likely sources of much of the radiation in the 1-5 MeV range based on some necessarily tentative measurements by Hall *et al.* (*Proc. 14th Int. Conf. Cosmic Rays, Munich*, **1**, 84; 1975) for the radio galaxy Cen A and arguments were put forward for Seyfert galaxies also contributing considerably, particularly above 100 MeV. At that time no Seyfert galaxies had been detected but now the subject has moved on and definite measurements have appeared. The way has therefore opened for a quantitative approach.

Schönfelder (this issue of *Nature*, page 344) has drawn attention to the recent observation by his colleagues and himself (in the press) of gamma rays from the Seyfert galaxy NGC 4151. The measurements show strong evidence for a rapid change of spectral

slope at about 3 MeV, the exponents being -1.0 at lower energies and -3.0 above. Such a coincidence with the energy for the shoulder in the diffuse spectrum as a whole is most interesting and Schönfelder goes on to see how the absolute intensity for summed Seyfert galaxies over the whole of space compares with the intensity of the diffuse background. If all X-ray Seyferts are like NGC 4151 then agreement is achieved within a factor of two and the circumstantial evidence for a summed discrete source for the shoulder is rather strong.

Clearly, measurements on more Seyferts are necessary and these should be available quite soon; in the meantime it appears to be unwise to put too much money on the baryon symmetric big bang cosmology if, in fact, its main prop is the gamma-ray shoulder. □

Cancer chemotherapy: *in vitro* test

from B. W. Fox and T. M. Dexter

THE increasing success of cancer chemotherapy is real but humans and their tumours vary so widely that it is not surprising that the sensitivity of tumours to therapeutic drugs also varies equally, even within apparently phenotypically identical tumours. Clearly, a pre-indication of the spectrum of sensitivity of a specific human tumour cell population to a range of possible antitumour agents could assist in deciding on the treatment required. To this end, many investigators have attempted to establish *in vitro* systems for the analysis of tumour cell populations and, in particular, the response of individual patients' tumour cells to the variety of chemotherapeutic agents available. Certain criteria need to be met: the test must distinguish between the growth of normal and tumour cells and the results need to be obtained sufficiently quickly to allow clinicians to respond to the data. Such a crude approach will not take into account differences in host conversion and detoxification efficiency for the drug, tissue specific accumulation, immunosuppressive activity effects, and many other host-associated parameters; but such *in vitro* tests would at least supply

an initial indication of relative effectiveness against a specific human tumour cell population.

A major criticism of the efficacy of *in vitro* systems for determining tumour responsiveness to chemotherapeutic drugs, has been the low plating efficiency observed for many tumour cells. However, most tumours consist of a heterogeneous population of tumour stem cells (which possess extensive, if not indefinite, proliferative ability) and other cells showing little or no proliferative activity—presumably representing more differentiated states or genetically sterile populations. Successful treatment of tumours, therefore, is based upon elimination of stem cells with reproductive ability, which represent only a fraction of the tumour cell population. Ideally then, an *in vitro* system for the response of tumour cells to various drugs, should be based upon a tumour clonogenic system where the response of cells with extensive proliferative ability is selectively measured. Such an assay has been developed by Anne Hamburger and Sydney Salmon at the University of Arizona in Tucson (*Science* **197**, 461; 1977) especially for multiple myeloma (*J. Clin. Invest.* **60**, 846; 1977) in which the tumour burden *in vivo* can also be measured more accurately and a more meaningful correlation with *in vitro* tumour stem cell colony formation obtained.

The problem of patient selection and the necessity of making a chemotherapeutic decision before results of such tests become available has meant that comparisons have to be retrospective. A study of this type has recently been reported (Salmon *et al.* *New Engl. J. Med.* **298**, 1321; 1978) where nine patients with myeloma and nine with ovarian cancer were treated with standard chemotherapeutic agents and their tumour cells simultaneously examined for their pattern of sensitivity against a range of standard agents. The not surprising result of these *in vitro* studies was the relatively large differences in sensitivity in otherwise histologically identical tumour types. Within these 18 patients, it was possible to correlate the results obtained *in vitro* with the subsequent outcome of the treated patients. In this limited study no patient who was resistant *in vitro* was sensitive *in vivo* and the majority who were sensitive *in vitro* showed a good response to the drug *in vivo*. Drug resistance towards bleomycin was also predicted from the *in vitro* assays during relapse after initial treatment with the drug.

The authors rightly point to the problems in drawing direct conclusions on the potential value of this test in practice as the "logistics and time constraints" of this type of assay could pre-

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sent serious ethical and economic problems. The measurement of cell colony formation and survival, the only legitimate end point, is irrevocably time consuming. However, as further retrospective and prospective correlation data accumulates, the stem cell assay time of two to three weeks may provide a stimulating field of research designed to identify parameters which could be used to shorten the time involved. □

Rejecting ophiolites

from Peter J. Smith

LINEAR marine magnetic anomalies must be due to magnetised material beneath the ocean floors. But that is to put the matter at its simplest and least instructive. What is the composition of the suboceanic material? How thick is it? How deep is it? How strongly magnetised is it? And what are the shapes of the magnetised segments that account for the alternating pattern of positive and negative anomalies? Taken together, these questions define a major problem—and one that must be solved if the correlation between marine anomalies and reversals of the geomagnetic field is to transcend mere phenomenology.

One approach to the problem is to model the anomaly-producing oceanic lithosphere; but however well a model predicts the observed anomalies it cannot provide a unique solution, still less one likely to correspond with reality. Nor can it provide a complete solution. The only way of doing that is to sample the oceanic lithosphere. In practice, however, direct sampling has produced only an incomplete, and thus confusing, set of data because the Deep Sea Drilling Project (DSDP) has thus far achieved a maximum penetration of only 0.6 km into the suboceanic igneous basement. Two recent expeditions to the mid-Atlantic ridge, for example, have obtained magnetic data suggesting that a magnetised layer of 500–1,000 m may be sufficient to account for the anomalies there. Away from spreading centres, on the other hand, samples are usually much less intensely magnetised, cores sometimes contain material magnetised in a sense opposite to that necessary to produce the observed anomaly, and the igneous column is often contaminated with sediment and voids. Magnetic layer thicknesses of up to 2.5 km or more are

therefore apparently required to account for some observed anomalies.

Presumably time and money will ultimately allow direct sampling to answer all relevant questions. In the meantime, however, some workers have suggested that a solution may be sought in ophiolite complexes, those sequences of rock types widely supposed to represent oceanic lithosphere now upthrust on to land. It is an enticing thought, for if such speculations about their origin are correct, ophiolite suites at present offer the only direct access to deeper sections of the oceanic lithosphere. But as Levi *et al.* (*Geophys. Res. Lett.* **5**, 473; 1978) now point out, there are dangers in taking so simplistic a view. For one thing, ophiolites are obviously older than they were when part of the *in situ* oceanic lithosphere, and some are older than any existing ocean floor. Second, it is by no means clear whether they represent lithosphere as formed at a spreading axis, as modified in fracture zones or as altered by subduction processes—or whether all three types exist. Then again, many ophiolite suites have been altered to a greater or lesser extent. But when did the metamorphism take place—in the original oceanic environment, during upthrust on to continent, or since this emplacement occurred?

As far as magnetic studies are concerned such matters are of vital importance, for magnetic minerals are very sensitive even to moderate heating for very short periods. Levi and his colleagues therefore suggest that before too many workers uncritically attribute the magnetic characteristics of ophiolite complexes to inaccessible parts of the oceanic lithosphere it would be useful to have a set of criteria by which ophiolites might be deemed suitable or unsuitable for modelling the magnetic properties of the layers responsible for marine magnetic anomalies.

Because the DSDP has only achieved a 0.6 km penetration of the igneous lithosphere, direct comparison between the present oceanic lithosphere and ophiolites can only be carried out in respect of the latter's upper pillow basalts and associated flows. Levi and his coworkers propose, therefore, that if an ophiolite complex is to be used to model the magnetic properties of the oceanic lithosphere, at the very minimum the magnetic characteristics of the complex's upper extrusives should correspond closely to those of submarine basalts from today's oceans. Specifically, this means that the extrusives should have Curie points in the range 100–459 °C, intensities of magnetisation $> 10^{-4}$ gauss, ratios of remanent to induced magnetisation > 1 , and no alteration beyond zeolite facies metamorphism.

These are, of course, necessary, but not sufficient, conditions. Even if the minimum criteria were satisfied there would be no guarantee that any given ophiolite complex would be completely suitable. Nevertheless, the application of just the minimum conditions has a devastating enough effect, as examination of six ophiolite suites shows. The Vourinos complex of central Greece is eliminated straight away because its pillow lava sequence is missing. The Smartville (California) complex has pillow lavas but they conform to none of the four criteria. The Oman Massif and Othris (Greece) complexes are more difficult to interpret but disobey at least one criterion. The only complexes conforming to all four criteria are those from Macquarie Island and the Troodos Massif (Cyprus). All of which suggests that, magnetically speaking, ophiolites may not be quite the help they once appeared. □

NATURE

A hundred years ago

THE medical students of Paris have not forgotten that Rousseau was a botanist as well as a philosopher, and sent a delegation on July 2 to Ermenonville to celebrate the 100th anniversary of his death. Three addresses were delivered in the name of the medical body—one by Dr Bergeron, the toxicologist, the second by M. de Lannesau, and the third by M. Baillon, himself a botanist and a professor of the School of Medicine. The speakers referred in eloquent terms to the love of Rousseau for nature, his observational genius, and his works on botany. The students had prepared a splendid crown made of *pervenches* (periwinkle, *Vinca*) the flower which Rousseau loved best, and which had been collected by them in the very forest where the philosopher spent his last years. As no boat was to be had to reach the island where the author of the "Nouvelle Héloïse" is buried, one of the students threw himself into the water and swam with the testimonial to the spot sacred to the memory of the impulsive Frenchman.

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review article

Mutator genes—pacemakers of evolution

James N. Thompson Jr* & R. C. Woodruff†

*Initially a genetic oddity, male recombination in *Drosophila melanogaster* is now being viewed as a means of detecting mutator activity and chromosome breakage in hybridising populations and is causing us to reconsider the source and rate at which genetic variability may be generated in nature.*

NATURAL populations contain large amounts of genetic variability. This variability provides the genetic basis for long-term adaptation and may even contribute to short-term physiological flexibility. Thus, it might seem strange that genetic factors that induce mutations or otherwise modify the rate at which variability is produced in natural populations have received comparatively little attention¹; strange, that is, until one considers the technical difficulties in detecting and measuring mutator activity in nature.

It is within this framework that one can begin to appreciate the growing attention being paid to what might otherwise be considered a genetic oddity. For decades, one of the first facts taught to beginning students of genetics was that there is no recombination in the males of *Drosophila melanogaster*. But in 1971, Hiraizumi² reported that a proportion of the males from a wild population in Texas showed low levels of recombination when heterozygous males were testcrossed to homozygous marked females. The amount of recombination was significant, but much lower than that typically observed in heterozygous females (for example, only 0.5% recombination in the *T-007* male recombination line between markers more than 50 map units apart as judged by recombination frequencies in females). Others began to look for recombination in *D. melanogaster* males and found it.

But even more importantly, studies of these male recombination (MR) lines soon showed a variety of correlated genetic events, including chromosome breakage and mutator activity. Thus, the ease of identifying mutator lines on the basis of recombination, and their world-wide occurrence, has focused attention on male recombination activity as an ideal experimental system to analyse the induction of both genic and chromosomal mutations and their impact on the genetic structure of populations.

Recent studies of male recombination and associated mutator activity revolve around three complementary questions. First, how widespread is it in natural populations? Is the male recombination and mutator activity syndrome an aberrant behaviour of one Texas population of *D. melanogaster* or is it more common than 70 years of *Drosophila* genetics would lead us to suspect? Second, what causes male recombination and its related genetic phenomena? This question can be asked at two levels. What is its precise genetic basis, and what events can stimulate it or modify its occurrence? Finally, what impacts do this and other mutator factors have on the level and type of variability in natural populations?

Distribution of MR activity

One of the first facts to attract general attention to male recombination was its surprising frequency in natural populations world-wide. Although it has not been detected in all populations, the initial report by Hiraizumi was soon followed by reports of male recombination in *D. melanogaster* collected from many parts of the United States³⁻⁸, Australia^{9,10}, England⁷, Greece¹¹, Israel¹², Japan¹³, Taiwan¹³, and Yugoslavia¹³. In addition, chromosomes from MR lines have been cited in association with mutator activity in flies from the USSR¹⁴ and other areas.

Wide distribution is not, however, the total picture. Male recombination can be extremely common within a population, being found in up to 100% of all wild males tested⁷. In addition, other species, including *D. ananassae*^{15,16}, *D. simulans*¹⁷, *D. subobscura*¹⁸, *D. virilis*¹⁹, and *D. willistoni*²⁰ also show spontaneous recombination in males, although the mechanisms may vary.

This immediately leads us to ask whether male recombination is a phenomenon recently affecting *D. melanogaster* or whether it has always been present, but simply not recognised. There is, in fact, good evidence that even Muller²¹ in 1916 and Bridges and Morgan²² in 1919 observed male recombination.

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In one of the first backcrosses performed with linked autosomal loci, for example, Bridges and Morgan²² observed a number of apparent recombinants when heterozygous males were crossed to homozygous marked females.

Why, then, was male recombination not reported from later experiments? It is certainly true that the amount of recombination per male is not large, and, although apparently common, not all populations necessarily show it. In addition, male recombination is generally absent in stocks that have been in the laboratory for a number of years, though the reason for this is not known. Presumably, when observed, as it must have been from time to time, later examples of male recombination were simply attributed to mutation or contamination and generally disregarded. With frightening certainty, the history of science teaches us that the facts we observe are sometimes dictated by what we expect to observe.

Hybridisation and MR phenomena

The real importance of male recombination, however, lies not in the recombination itself, but rather in the genetic events with which it is correlated and for which male recombination is conveniently diagnostic. These correlated genetic events share one key trait as a consequence of their detection. All are stimulated by outcrossing or hybridising an MR line with another stock.

The evidence that we shall discuss below strongly suggests that these MR-related events are not common, or do not even occur, in the base populations from which the chromosomes are taken; they are only expressed on outcrossing. In recognition of the importance of hybridisation, the term 'hybrid dysgenesis'^{10,25} has been coined for the syndrome of events associated with male recombination. The relationship between mutator activity and hybridisation has profound implications for our understanding of the origin and significance of genetic variability. But before discussing some of these implications, it would be wise to outline our present knowledge of the components and causes of hybrid dysgenesis.

One of the first things Hiraizumi² noticed was that the paternal MR chromosome is commonly transmitted by heterozygous individuals at frequencies considerably less than the mendelian expectation of 0.5. The observed transmission frequency² (k) varies from about 0.30 to nearly the control values of 0.50 or higher in different lines and is negatively correlated with the amount of recombination^{2,23,24}. As confirmation of this relationship, an extensive study of lines artificially selected by the Kidwells²⁵ for increased and for

decreased male recombination showed that k was significantly higher (that is, nearer normal) in the low recombination line than in the high selection line. Not all MR stocks show this marked distortion⁷, however, and the relationship between MR-generated distortion of segregation ratios and the classic segregation distorter system²⁶ is not known.

MR chromosomes are also associated with very potent mutator activity. A five- to tenfold increase in X and second chromosome lethals has been detected from a number of different male recombination lines^{23,27-30}. Although no clear locus-specificity has been found in the lethals generated by these mutator lines, Slatko and Hiraizumi²³ noted a slight grouping of lethals near the tip of the X chromosome. Small clusters of allelic lethals have also been found in most of these studies and in our unpublished work. Clusters of mutant-carrying sperm could reflect either premeiotic (mitotic) mutator action, a possibility referred to in other contexts below, or high

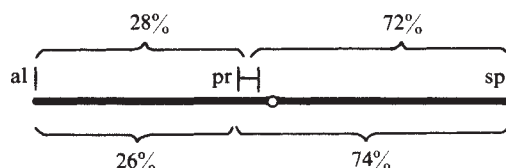


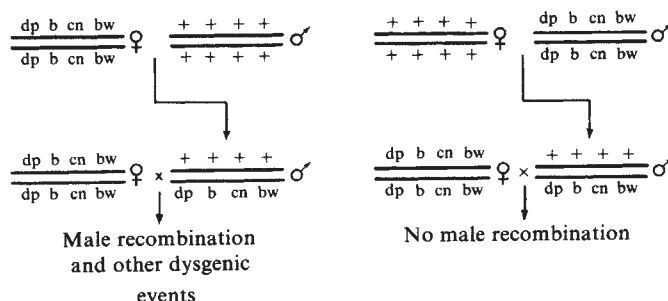
Fig. 2 A comparison of the distribution of male recombination exchange points (above the chromosome) and unique aberration breakpoints (shown below the chromosome) for the second chromosome of *D. melanogaster*. Cytological subdivision of breakpoints parallel to the genetic division was accomplished by pooling breakpoints in chromosome sections 21–35 on the left and 36–60 on the right. The recessive markers are *aristless* (*al*, 0.0), *purple* (*pr*, 54.5), and *speck* (*sp*, 107.0). (Redrawn from ref. 3.)

levels of locus-specific mutator activity similar to that reported for X-linked visible mutations by Green and collaborators^{12,14}. Thus, the question of locus-specific mutator activity is still an open, and clearly important, one.

A third component of hybrid dysgenesis is sterility among F_1 progeny from crosses between MR lines and standard laboratory strains^{6,7,10}. Sterility frequencies also demonstrate another key feature of MR activity that is common to recombination and other (but not always all²⁸) manifestations of hybrid dysgenesis. Frequencies show a reciprocal cross effect^{6,7,10,29,31}, in that they are dramatically higher when the male, rather than the female, parent is from the MR strain (Fig. 1). Indeed, in most instances, the traits are only found in those crosses in which the MR chromosomes are paternal in origin. This difference between reciprocal crosses has been the basis for a number of hypotheses concerning the nature of MR suppression and activation, including possible interactions between MR chromosomes and the maternal cytoplasm⁶ perhaps involving a virus or other microorganism^{3,4,7}, spatial organisation of chromosomes¹⁰, and X-linked suppressors of MR activity (Y. Hiraizumi and B. Slatko, personal communication).

But probably the single greatest clue to the nature of hybrid dysgenesis was the observation that so-called 'unique' chromosome rearrangements (that is, novel rearrangements that are individually rare) are often detected in populations showing male recombination activity and in outcrosses from those populations^{3,5,8,13}. In addition, the sites of recombination and the chromosome rearrangement breakpoints are often highly correlated (Fig. 2). It was quickly recognised that unique chromosome rearrangements, male recombination, sterility, distortion of segregation ratios, mutator activity, and perhaps even non-disjunction³³—in short, the entire MR syndrome of

Fig. 1 The events of hybrid dysgenesis show a marked reciprocal cross effect, in that crosses initiated with males from many natural populations will produce heterozygous males that undergo mutation and chromosome breakage (left), whereas the reciprocal cross leads to no dysgenic traits.



hybrid dysgenesis—could be explained on the basis of chromosome breakage as the primary event^{3,7,13,23,24} (Fig. 3).

This hypothesis was tested directly³⁵ by analysing spontaneous chromosome breakage during male meiosis in reciprocal crosses between the MR strain *OKI* and laboratory stocks. Chromosome breakage was totally absent in the *OKI* base stock itself—an observation of central importance when considering hybridisation as a key event. On outcrossing, however, a clear reciprocal cross difference was found. Chromosome breakage was detected only in those male progeny from crosses in which the parental male was from the *OKI* line. Although meiosis was completely normal in control crosses and in crosses in which the MR-line parent was female, outcrossing males from *OKI* produced male progeny whose spermatocytes had anaphase bridges at both first and second meiotic divisions (Fig. 4). These were commonly accompanied by more than the single fragment expected from conventional cases of inversion bridges and fragments. In extreme instances, one or more of the autosomes were completely pulverised.

These observations suggest that hybrid dysgenesis in *OKI* is essentially a meiotic phenomenon, though some premeiotic primer event or limited amounts of premeiotic recombination may occur, for example, and premeiotic events in other MR lines seem well-documented^{28,38}. The lack of mitotic chromosome breakage in *OKI* seems inconsistent with the occasional small clusters of recombinants in *OKI* crosses, but it is confirmed by the absence of increased somatic recombination in outcrosses³⁶ and by the absence of chromosome breakage in mitotically active larval brain cells³⁷. Thus, these

Fig. 3 Possible consequences of chromosome breakage in outcrossed natural population lines. A wide diversity of genetic events can theoretically be attributed to chromosome breakage. (After ref. 34.)

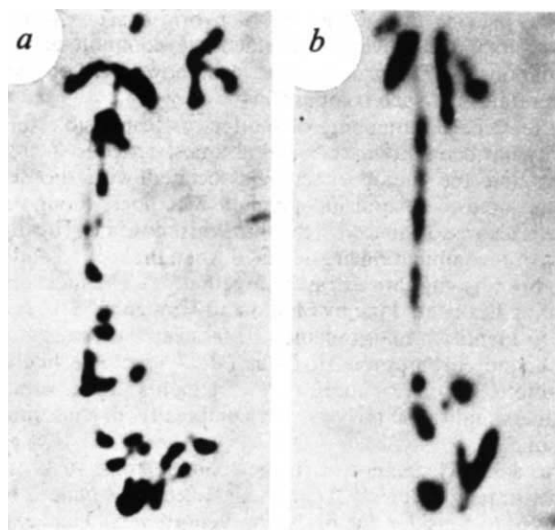
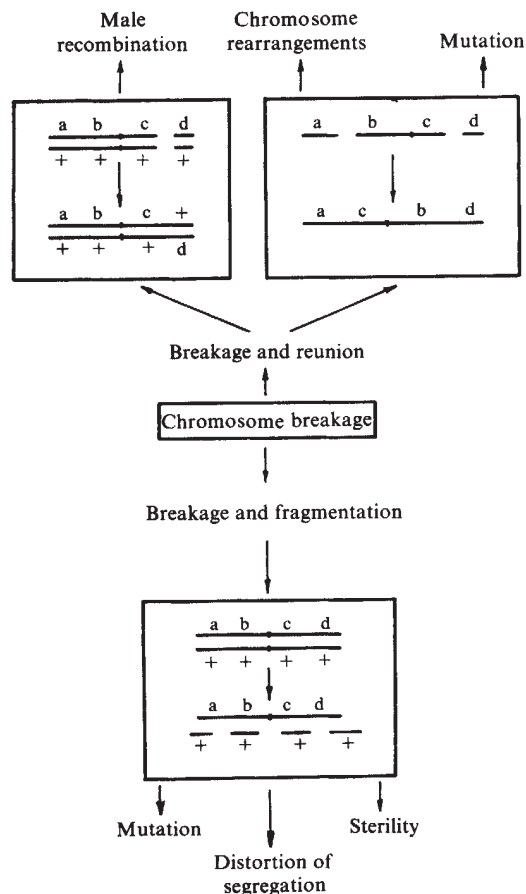


Fig. 4 Bridges and/or fragments are found in up to 20% of the anaphases in spermatocytes of outcrossed *OKI*, a MR strain of *D. melanogaster*. In some instances one or more chromosomes can be completely pulverised. (From ref. 35.)

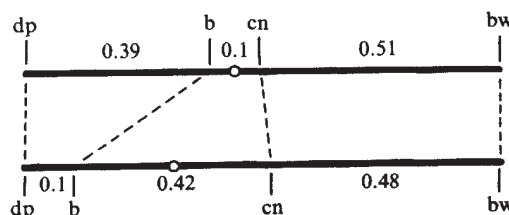
observations fuel the controversy that has surrounded the detection and interpretation of clusters of recombination events since the analysis of MR lines began.

Mapping of MR factors

Although we believe the events of hybrid dysgenesis to be primarily meiotic in origin, at least in some MR strains, male recombination is clearly different from normal female recombination in many ways. One of the most striking differences is in the relative distance between pairs of loci as summarised in the female-derived and male-derived maps (Fig. 5). On the second and third chromosomes, male recombination is more frequent around the centromere and on the right arm^{3,7,28,38}. Indeed, the male-derived map corresponds most closely with the mitotic recombination and salivary gland chromosome maps³⁹, which further clouds the mitotic/meiotic paradox. The paradox disappears, however, if we assume that MR events are physically induced at random along the chromosome, yielding a map correlated with amount of DNA or with salivary gland chromosome band distances.

Map differences are also reflected in modified female recombination frequencies³¹ in appropriate reciprocal MR-line

Fig. 5 A comparison of the female-derived recombination map (above) and the male-derived map (below), illustrating that a greater proportion of recombination in males occurs around the centromere and in the right arm of chromosome 2 in *D. melanogaster*.



crosses, proving that the effects of hybrid dysgenesis are not limited to males. Inversions, which are common in natural populations of *D. melanogaster*, can, however, confuse the interpretation of such comparisons²⁸.

At least partial mapping of the factors responsible for MR activity has been attempted several times. There is strong evidence that the major effect is associated with the second chromosome in several independent MR lines, though some effect is also associated with the third chromosome. The highest levels of recombination are detected when the second and third MR chromosomes are extracted together^{3,4,7}. The most elegant mapping has been done by Slatko and Hiraizumi⁴⁰. They were able to identify a major element (*Mr*) near the centromere of the second chromosome of their *T-007* line. The likelihood that there are one or more major elements plus a variety of modifiers indicates the genetic complexity of this mutator system.

The acknowledgment of further complexities, such as sensitivity to temperature^{11,33} and to age of the MR male³³, finally brings us to consider one of the most controversial and exciting aspects of hybrid dysgenesis. There is growing, but still annoyingly ambiguous, evidence that the factors responsible for MR activity can be transmitted by non-genetic means. Sochacka and Woodruff⁴¹ and Slatko and Hiraizumi (unpublished) have been able to induce male recombination in laboratory stocks by injecting whole fly homogenates of their MR strains, although similar experiments with other MR lines have not always been successful. This, together with some evidence that MR activity can be transmitted by feeding MR-strain homogenates (ref. 42 and J. N. T., C. M. Smith and R. C. W., unpublished), suggests that the ultimate cause of hybrid dysgenesis may be a micro-organism or similar transmissible agent. Thus, genetic mapping experiments might be identifying either modifier genes or foreign genetic elements inserted into the genome (see ref. 12).

There are certainly precedents for such an hypothesis. Hybrid sterility in *D. paulistorum*⁴³ is caused by a mycoplasma,

and the chromosome pulverisation we have seen is similar to that induced by viruses^{35,44}. Although the nature of the factor is not yet clear, a virus is probably the most widely favoured contender. From the properties of the visible mutations induced by MR lines, Green¹² suggests that they are due to the insertion of a genetic element analogous to bacteriophage Mu or insertion (IS) elements.

The need to generalise accurately about an initial cause of hybrid dysgenesis immediately raises another very difficult question. How do we know that the expressions of hybrid dysgenesis in the diverse populations of *D. melanogaster* and other species world-wide are being caused in the same way? Although the evidence is strongly in favour of a common cause in some instances, the truth is that we do not know whether all MR lines contain the same elements—genetic or micro-organismal. Indeed, if MR activity is genetically suppressed in different ways in various natural populations, the similarity of MR factors may be difficult to determine short of isolating them strain by strain.

Probably our best approach at the moment is to compare the characteristics of various MR strains and other mutators that might be similar. This is done in Table 1. Although the list is not exhaustive, we have attempted to include the majority of mutator factors in *D. melanogaster* and a sample of other factors that show some of the components of hybrid dysgenesis.

One striking thing about the table is that, although a fair number of mutators have been identified, much important information is missing. In fact, one of the most valuable contributions of recent studies of MR lines is the outline of a syndrome of characteristics and a causal hypothesis that can guide future studies of potentially related phenomena. For example, many studies of female sterility in flies outcrossed from natural populations^{46,47} show correlations with phenomena such as cytoplasmic influences⁴⁸ that suggest they might be MR strains. An episome-like factor in *D. robusta*⁴⁵ breaks chromosomes and shows many similarities to the *D.*

Table 1 Comparison of genetic events, including mutator activity, associated with hybrid dysgenesis involving strains of *Drosophila melanogaster* from natural populations

Strain and origin	Mutator activity			Sterility	Male recombination	Modified female recombination	Distortion of segregation	Non-disjunction	Refs*
	Induction of lethal mutations	Induction of visible mutations	Induction of chromosome aberrations						
<i>hi</i> (Florida)	+	+	+		—†		—†		63
<i>mu-F</i> (Florida)	+	+	—						66
Florida	+	+	+		+				3
<i>Mr</i> (Texas)	+	+			+	+	+	+	2
<i>OKI</i> (Oklahoma)	+		+	+	+		—		7
Eastern USA	+			+	+	+	+	+	6
<i>AW, JH</i> (Pennsylvania)			+		+				5
N. Carolina	+		+		+				8
N. Carolina	+		+						32
<i>MRF</i> (Ohio)					+		+		4
<i>SD</i> (Wisconsin)	+	+	+			+	+		26
<i>RD</i> (unknown)			+				+		68
Texas; Mozambique				+					47
Europe; Africa; W. Indies				+					48
<i>31.1</i> (Greece)	+		+	—	+		+		11
<i>OYW</i> (Yugoslavia; Japan; Taiwan)	+		+		+				13
Haifa, Israel	+	+			+				12
USSR		+		+					14
<i>delta</i> (Japan)	+	+			+	+			55
NSW, Australia	+			+	+		+		10

* Only one representative entry of the considerable literature is given for each factor.

† Tested about 30 years after *hi* was isolated⁶⁸.

melanogaster system. Natural populations of several species have been observed to contain 'sex-ratio' factors⁴⁹⁻⁵², some of which break chromosomes and can be transmitted by injection⁵³. Hybrid dysgenesis might even be involved in some 'meiotic mutants' isolated from natural populations⁵⁴. On the other hand, there are also characteristic differences among MR-type strains (see ref. 55), warning us to avoid applying these ideas too rashly to all traits.

Mutator factors in evolution

Studies of MR lines have not only suggested a common cause for a wide variety of phenomena in natural populations, they have also stimulated a reconsideration of our ideas about the rate of mutation in natural populations and the role of mutator factors in evolution.

It has long been recognised that mutation rate is, to some extent, modifiable by the genetic background. Most changes in the genetic environment can be expected to increase the mutation rate of a gene⁵⁶. One hypothesis is that in a population within a typically stable environment, selection acts to minimise the mutation rate. Support for this hypothesis comes from the observation that changes in the genetic system, such as shifts from inbreeding to outbreeding⁵⁶ or hybridisation between species⁵⁷ can lead to a dramatic increase in mutation frequencies. This makes particular sense if one considers that the genetic basis of the suppression of mutation is likely to vary from population to population, and changes in breeding structure or hybridisation would disrupt coadapted suppressor gene complexes. Such a view was first proposed by Sturtevant⁵⁷ and has been discussed in a number of ways, such as the potential mutator hypothesis of Cardellino and Mukai⁸ and earlier by Ives⁶³.

We believe that the hybrid dysgenesis syndrome is caused by the disruption of genetic suppression of mutator activity through hybridisation between populations. This, in turn, can lead to an explosive increase in mutation (on the order of a 10-fold increase in a set of experiments comparing mutation frequencies before and after hybridisation (R. C. W. and J. N. T., unpublished)) and to the production of chromosome aberrations. Indeed, if one can extend the term far enough, the phenomenon of hybrid dysgenesis is probably very widespread. Cytoplasmic incompatibility in *Culex* and *Aedes* mosquitoes^{58,59}

is one well-documented example that sounds very much like the hybrid dysgenesis described in *Drosophila*. This incompatibility is apparently caused by a rickettsia-like micro-organism. Chromosome fragmentation has often been described in interspecific hybrids^{57,60,61} and has been cited as an isolation barrier in some species groups⁶².

One consequence of our hypothesis of explosive mutation events resulting from hybridisation between populations is that most current estimates of mutation rates in natural populations are probably too high. These estimates generally involve matings between wild flies and laboratory populations—an innocent act that, in itself, may generate mutations. Supporting evidence comes not only from laboratory work, but also from assays of mutation in wild populations. For example, during an 8-year study, Spencer⁶⁴ found that the frequency of visible mutations reached two peaks of about 30 months, separated by a 3-year period of no visible mutation. Thus, there appeared to be times of explosion and times of mutational drought.

In summary, studies of the mutator activity associated with male recombination lines have suggested that a variety of genetic events in natural populations may be causally related—perhaps even associated with the interaction of a micro-organism and the host genome. Little significant mutation may normally occur within a stable population. It may be limited to periods of mutator gene activity induced by breakdowns in genetic suppression, due to hybridisation or to the genetic consequences of selection in response to environmental stress. In extreme instances, it may even contribute to isolation, by reducing gene flow between hybridising groups through sterility and chromosome damage. During times of stability, however, selection acts, not to eliminate these mutator factors, but to suppress their effect. This hypothesis clearly differs from the typical view of mutation activity in natural populations. Further work may confirm that, as Neel⁶⁵ wrote in 1942, without exaggeration, mutator genes may be the pacemakers of evolution.

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articles

Computed crystal structure images for high resolution electron microscopy

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It is now possible to compute images of crystal structures corresponding to experimental images produced by using high-resolution transmission electron microscopy (HRTEM). The variation of contrast with crystal thickness, microscope resolution and defocus has been faithfully reproduced by the calculations for some minerals and a binary oxide.

ONE of the more interesting recent developments in solid state science is the use of the transmission electron microscope to obtain high resolution images which show structure within the unit cells of synthesised crystals¹⁻⁴ as well as minerals⁵⁻⁹. It is now possible to obtain experimental images that appear to have a one-to-one correspondence with the structure as determined by X-ray or neutron diffraction. Interpretation of these high-resolution transmission electron microscopy (HRTEM) images has, on occasion, been a subject of controversy because a given crystal can produce a variety of images, depending on parameters such as the objective aperture used, the focus setting (defocus) of the objective lens and crystal thickness. The ability to produce calculated images of a given (or trial) structure makes it possible to resolve such controversies.

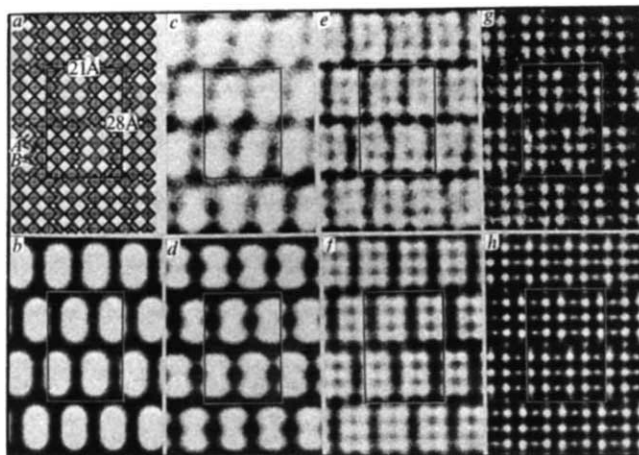
HRTEM images are sometimes chosen for publication on the basis of how closely their character matches the investigator's conception of how the crystal structure should appear. Such images are then often interpreted as projections of the potential or charge density within the crystal on the basis of either the Scherzer thin phase object (TPO) model¹⁰ or the Cowley-Moodie projected charge density (PCD) model¹¹. Commonly, such interpretations seem satisfactory, at least qualitatively, even though values of various image parameters such as crystal thickness may be well outside the ranges allowed by the approximations.

Computer programs have now been developed¹²⁻¹⁴ which can be used to calculate the image to be expected from a given model structure. These programs use the Cowley-Moodie multislice formulation¹⁵ to include dynamic diffraction effects and the effects of specimen parameters like orientation and thickness. They also consider the effects of microscope parameters like accelerating voltage, objective lens defocus and aberrations, and the finite illuminating angle produced by the condenser lenses. With the use of these programs, problems of ambiguity in interpreting HRTEM images can be minimised by computing the image for a variety of operating conditions

(especially defocus) and obtaining matches of these computed images with experimental ones (M. A. O'K. and P. R. B., in preparation).

Calculations have already shown^{16,17} that images from very thin regions can be interpreted in terms of the PCD and TPO approximations. However, as crystal thickness increases, the images change from TPO or PCD-type images to ones which, while still qualitatively representative of the structure, cannot be interpreted quantitatively in terms of either the TPO or PCD approximations. Many published images apparently come from such thicker regions. As it is possible to obtain many different images, all having the same general character, it is desirable to designate all such images by the same name. We use the term 'crystal structure image'¹⁸ as applying to those images which have the qualitative character of TPO or PCD

Fig. 1 Structure images of Nb₁₂O₂₉. *a*, The structure consists of blocks of corner-sharing octahedra (3 × 4) containing large (3.8 Å) tunnels (A). The blocks are joined by shared octahedral edges, producing small tunnels (B). The unit cell is outlined. *b*, Structure image computed for 10 Å resolution showing each block of octahedra as one large white spot. At 7 Å resolution, each spot tends to split in two in both the experimental (*c*) and computed (*d*) image. The 4 Å resolution image shows white spots corresponding to the large tunnels (A) between octahedra (*e*, experiment; *f*, calculation). At 2.5 Å resolution, the small tunnels (B) are revealed as smaller white dots. The experimental image (*g*) was obtained using a microscope with an accelerating voltage of 1 MV; the calculated image (*h*) was computed for this voltage.



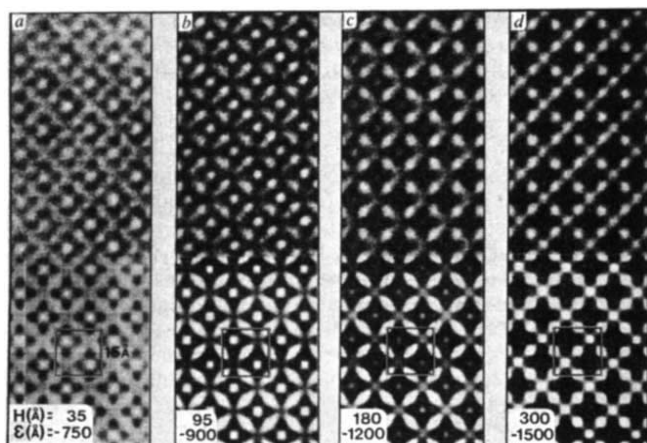


Fig. 2 Experimental (upper) and calculated (lower) images from a crystal of the mineral vesuvianite. The unit cell is outlined. Values of crystal thickness (H) and microscope defocus (ϵ) used in the computation are shown inset into each computed image. The original crystal was tilted, causing the thicker regions to be further out of focus than the thinner. For a thickness of 3 unit cells (35 Å) both the experimental and computed images show a projection of the crystal charge density (as predicted by the PCD theory) and are thus the ideal structure image at this resolution (4.5 Å). As crystal thickness increases, the image changes character until the white tunnel in the centre of each quaterfoil disappears.

images calculated at the same resolution. This term thus includes the very thin crystal images which match the TPO and PCD images quantitatively, as well as those images which still 'look like' the crystal structure even though obtained from slightly thicker regions of crystal. It should be noted that these types of images contain far more useful information regarding crystal structures than the 'lattice fringe' images, which reveal the periodicity of the lattice but not detail within the unit cell.

The character of the crystal structure image changes drastically with change in resolution. Figure 1 shows a series of experimental and calculated structure images of the 'block structure' $\text{Nb}_{12}\text{O}_{29}$. At 10 Å resolution (Fig. 1b) the image can be easily interpreted by inspection of and comparison with the structural drawing (Fig. 1a); the 10 Å × 14 Å white spots cor-

respond to the positions of blocks of metal-oxygen octahedra, while the black areas are regions which contain a higher density of metal atoms due to octahedra overlap. The computed image thus confirms Allpress' interpretations¹⁹ of experimental images obtained at this resolution from similar block structures. Calculations predicted that at increasing resolutions (6 to 7 Å) the large white spots would start to split in two (Fig. 1d). This type of crystal structure image is not intuitively interpretable, since it does not provide a one-to-one correspondence with the structure. Nevertheless, it has been reported (Fig. 7 of ref. 20) and an image is shown in Fig. 1c. Calculations confirm that it is a valid structure image of $\text{Nb}_{12}\text{O}_{29}$ and its occurrence in a specimen is not due to the presence of defects or of some other phase.

Calculations at 4 Å resolution (Fig. 1f) match the experimental image (Fig. 1e), which is a significantly improved version of that obtained by S.I.²¹. The group of six large tunnels between the octahedra in each block is seen as white spots. Our calculations predicted that at resolutions of around 3 Å the small tunnels between overlapping octahedra (Fig. 1a) would become visible (Fig. 1h), so that each individual octahedron would appear as a black dot. Such a structure image (Fig. 1g) has recently been published²².

Since many experimental HRTEM images are obtained in our laboratory, we are currently using imaging calculations to aid in their interpretation and to check interpretations made before sophisticated computer programs were developed. Figure 2 shows experimental and calculated crystal structure images of the mineral vesuvianite (the structure is shown in Fig. 7 of ref. 18). Calculations show that only at the thin edge does

Fig. 4 Through-focus series of images of a crystal of $\text{Nb}_{12}\text{O}_{29}$. Computed images are shown as inserts in the lower right of each experimental image. Values of defocus are marked. While the image at -1,600 Å is more difficult to interpret than that at optimum defocus (-640 Å), it actually contains more information.

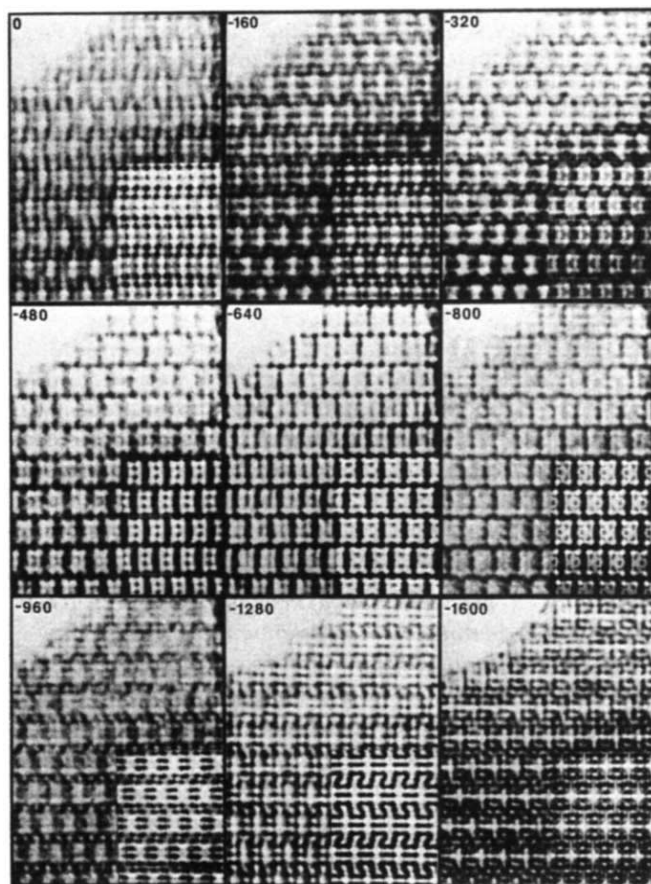
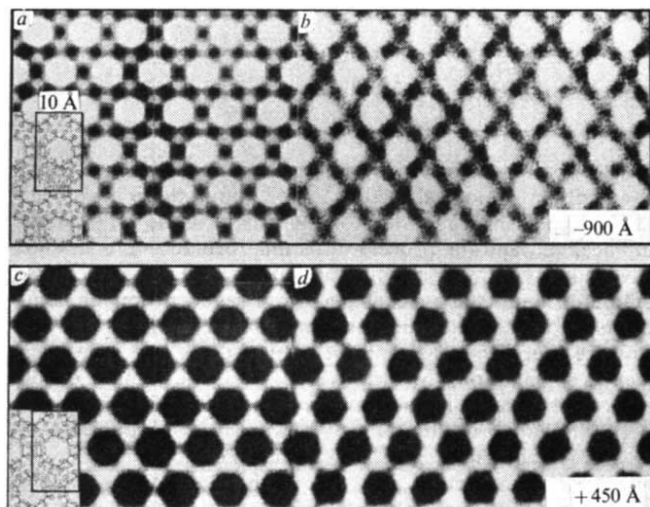


Fig. 3 Calculated (a, c) and experimental (b, d) images of the mineral cordierite in a - b projection. The structure image at defocus of -900 Å shows the large tunnels surrounded by a network of metal and oxygen atoms. At +450 Å defocus the contrast is approximately reversed, so that tunnel positions appear black. Drawings of the structure to the same scale and with one unit cell outlined are also shown.



the image correspond to the PCD-type image (Fig. 2a). As the crystal becomes thicker, the image changes but it remains recognisably a structure image over an appreciable range of thickness. Values of thickness were obtained by matching computed images with experimental images in single unit cell increments of thickness. Four of these matched images are shown in Fig. 2.

Once computed images are available then, apart from the sense of satisfaction in obtaining an image which looks like the structure, there is no *a priori* reason to prefer the optimum defocus image over images obtained at other values of defocus, especially since image character depends on both microscope resolution (Fig. 1) and crystal thickness (Fig. 2). Now that images can be computed, a through-focus series matched with a series of computed images is a much better test of the structure model used than any one particular match (say at optimum defocus). The images in such a series emphasise different spatial frequencies—that is, they test the contributions to the image of the various diffracted beams in many different phase combinations. In fact, because of finite incident beam convergence, images obtained at other than the optimum defocus may contain information at spatial frequencies greater than those frequencies present at the optimum defocus²³. For these reasons we routinely match 'through-focus series' of experimental images obtained from the same area of crystal.

Figure 3 shows how images from a crystal of the mineral cordierite orientated in *a-b* projection (Fig. 5 of ref. 18 shows the structure), can appear to reverse contrast with a change of focus. In fact, however, this is not an exact contrast reversal; the image showing black tunnels at +450 Å defocus has lost detail which is visible in the areas surrounding the tunnels in the -900 Å image. With more complicated structures and larger unit cells (and, of course, with higher resolution) the changes in image character with defocus are even more drastic. Figure 4 shows images of Nb₁₂O₂₉ over the defocus range 0 to -1,600 Å. The image at -160 Å is an approximate reverse contrast version of the structure image at -640 Å but the other images are more difficult to categorise. The structure image is not of sufficient resolution to reveal the small tunnels (B in Fig. 1a) but information about them can be seen in the higher resolution image at -1,600 Å defocus.

Computed images can thus be useful in predicting the character of the crystal structure image and, in particular, how

this character changes with microscope defocus, crystal thickness and image resolution. They can also be used to distinguish between several possible models for a crystal structure, or to identify defects within a known structure.

Although a relatively new technique, we believe that HRTEM will have increasingly wide application in the study of solids—in solid state physics, chemistry or mineralogy. The apparent ease of intuitive interpretation can produce problems because of the multiplicity of images that can be produced by a given structure. Computation of the images provides a quantitative verification of the intuitive interpretations and, indeed, proves to be necessary for a proper understanding of HRTEM images (M. A. O'K. and P. R. B., in preparation).

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Origin of lamination in deep sea, fine-grained sediments

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Many mechanisms have been proposed for the origin of lamination in deep sea sediments, but without general agreement. Experiments in other environments have suggested that several different processes may operate in particle flow conditions. Those relevant to deep water conditions are briefly discussed, and a new quantitative model developed for lamina formation in fine-grained sediments on a continental margin.

SILT laminated muds are very common marine facies and occur repeatedly in geological records¹⁻³. Although there have been many mechanisms suggested for their deposition, the mode of emplacement and the process of lamina formation are still not fully understood. Velocity fluctuations are, perhaps, the most commonly endorsed mechanism for the generation of lamination^{2,4-6} in marine sediments. Random and regular fluctuations are found in most types of natural flows and have been given as explanations for various types of stratification in eolian dunes⁷, for parallel lamination in delta plain sediments⁸, and micro-lamination in tidal muds^{9,10}. It has been postulated that the quasi-cyclic bursting process in turbulent boundary layers will give rise to vertical variation in the texture of sediment being deposited, thus defining a single horizontal lamina for

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each cycle^{11,12}. For fine-grained sediments, the time scales suggested are often too short to allow a mud layer to deposit¹³.

The concept of turbid layer flow has been introduced to explain the movement of mud and silt across the Californian continental shelf and down the slope¹⁴. One lamina would result from each of these long-period flows. Such turbid layer flows have subsequently been considered to be responsible for much of the fine-grained silt-laminated muds which are so common on outer continental margins^{15,16}.

A different view of the origin of lamination involves the congregational segregation of like particles near the base of a sediment-laden flow. At current velocities of 15–30 cm s⁻¹, silt and large muddy flocs, deposited from suspension, still move as bed-load; the flocs form cohesive patches increasing the rate of clay deposition from the base of the current until most of the clay is removed; silt deposition is then resumed¹⁷. Experimental studies in a circular flume¹⁸ demonstrated the formation of lamination in the absence of current pulsations. During a short period of traction or rolling as particles deposit from a uniform flow, the particles show a tendency to join stationary ones of equivalent size, shape and density. A very similar picture was deduced from observations on stratification in coarser grained deposits¹⁹.

Various authors have proposed lamina formation by the migration of bed forms^{7,20–22}. Experiments with sand at shallow flow depths²³ showed the formation of laminae during the migration of low-relief ripples and in-phase waves. When sufficient sediment was present for aggradation, the concentration of coarse material in the troughs buried the finer material on the stoss side of the underlying ripple. The migration of small current ripples with straight, long crests and muddy troughs has been observed to produce sand/mud laminae from a few cm to a few tens of cm in length²⁴. This produced flat, slightly lenticular bedding.

Other mechanisms proposed for the formation of laminae include differential settling and diagenetic effects²⁵; gyres in the tails of turbidity currents^{26,27}; a series of light suspensions floating at different levels behind a single, floor hugging turbidity current^{26,28}; and reflection of a turbidity current from the walls of a small basin²⁹. More significantly, internal lamination and cross lamination in cores from the western North Atlantic continental margin have been considered as evidence of reworking and placer concentration by contour-following, bottom currents. The association of abundant laminae in a rise environment with contour currents has become well established^{30–33}.

Sediments of the Scotian margin

The silt-laminated mud facies (Fig. 1) on the outer continental margin off Nova Scotia has been shown to be dominantly of turbidite origin; the evidence includes the inference of the direction of flow from the sediment fabric, the presence of systematic grading in the depositional units and the absence of bioturbation from these units³⁴. This throws considerable doubt on the hypothesis that similar facies found in other areas arise from either the action of contour currents or the flows of thin turbid layers. However, to appreciate the possible importance of turbidity currents in other environments a much clearer understanding of the depositional processes, particularly those producing silt lamination, is needed. A depositional model, as far as possible quantitative, is required.

For fine-grained sediments it has been proposed that the rate of deposition, R , of sediment of settling velocity, w , is given by an equation of the form,

$$R = \rho_s C w P \quad (1)$$

where C is the concentration of the sediment of settling velocity, w and density, ρ_s , in the flow just above the viscous sub-layer of the bottom boundary layer; P is the probability

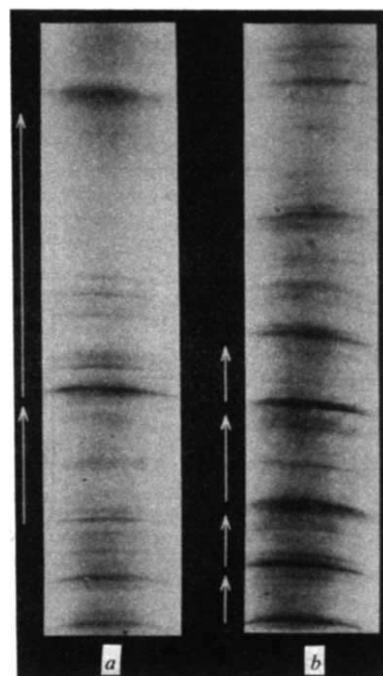


Fig. 1 X-radiograph prints of silt-laminated muds from the Nova Scotian outer continental margin. The width of the core sections is about 5 cm. The dark layers are silt laminae, and the arrows show probable graded laminated units.

that a given particle will be deposited³⁵. Data from experiments with fine, noncohesive silt³⁶, suggest that P is given by

$$P = \nu \left(1 - \frac{\tau}{\tau_c} \right) \quad (2)$$

where τ is the local bottom stress, $\tau_c(w)$, a critical value for the deposition of sediment of settling velocity, w , and ν is a factor to account for other influences on the probability of deposition. This is generally in line with the experiments on the deposition of cohesive material. A plot of τ_c as a function of w has been constructed³⁵, based on a wide range of data^{36,37}.

A layer of sediment of thickness q , and solid concentration λ , is therefore deposited in time T_0 , where

$$\lambda \rho_s q = T_0 \sum_w R(w) \quad (3)$$

the layer consisting of contributions from the various sediment sizes (of differing settling velocities) in suspension. Sediment analysis allows us to determine λ , to identify q as a layer with particular characteristics, and to obtain from its grain size distribution, $p_s(D)$, the proportion of grains within a particular size interval. There are two cases.

For silts, w is a well defined function of D , so that equations (1), (2) and (3) can be written in the form,

$$p_s(D) = \frac{C(D)w(D)\nu T_0}{q\lambda} \left(1 - \frac{\tau}{\tau_c(D)} \right) \quad (4)$$

For muds, there is no simple relationship between the grain size obtained by analysis of the deposit, and the dynamic behaviour of flocculated particles in suspension. However, a relationship between dispersed grain size distribution and flocculated particle size distribution has been suggested³⁸, and is supported by our experiments³⁴.

An important consequence of equation (4) is that there should be no silt deposited of grain size less than some critical diameter, D_c , given by: $\tau = \tau_c(D_c)$.

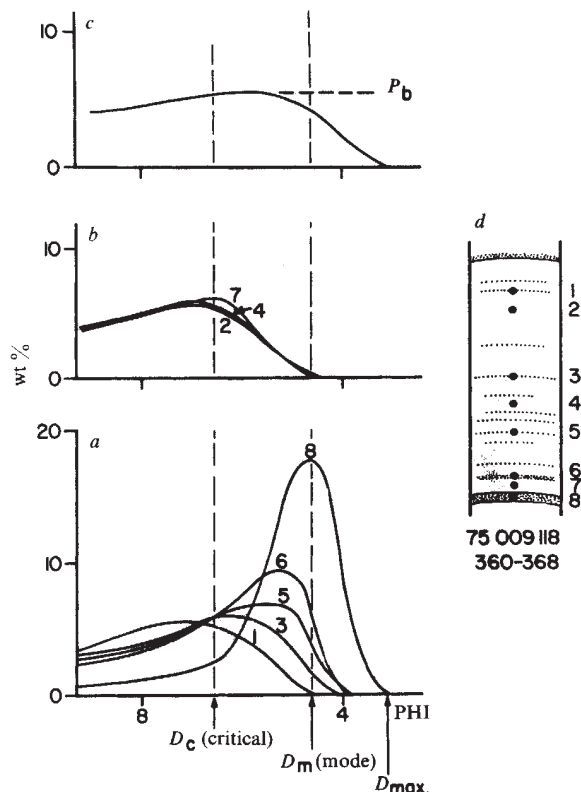


Fig. 2 Smoothed, weight-per cent, grain size curves from analyses of *a*, individual silt layers; *b*, mud layers; *c*, whole unit; and *d*, whole graded laminated unit shown as a schematic core section. The numbers beneath the core section are the core code and section depth in cm. The critical grain diameter (D_c), silt mode (D_m) and maximum silt size (D_{max}) are shown. See text for further discussion.

Figure 2 shows a sequence of size analyses through a typical graded laminated unit from a Scotian margin core. Proportional summation of these analyses, or summation of a continuous series of measurements through a silt-mud couplet, a silt lamina and the overlying mud layer, produces a fairly even distribution of grain sizes.

The size distribution of the silty layers shows a small quantity of very fine material, but a distinct change in trend at some intermediate grain size. This provides a first, crude estimate of D_c , and hence the actual stress, τ , at the time of deposition (Fig. 2). It is implicitly assumed above that there are finer particles in the flow not being deposited because τ_c is too large.

The size distribution of the superjacent mud layer shows that the coarse tail begins to fall off at the same size interval with which the silt curve changes to a marked upwards trend. This point, therefore, also provides a crude estimate of τ_c . The curve decreases to zero at the modal silt size (Fig. 2). This close match between silt and superjacent mud is, clearly, strong evidence that they were deposited almost simultaneously from the same flow. The flow is one in which the larger grains are behaving as individual silt particles during the depositional phase.

Knowing the stress τ , the velocity of the current U can be estimated as $\tau = c_D \rho U^2$ where c_D , the drag coefficient, has a value close to 2.5×10^{-2} for the flow over a smooth, fine-grained surface¹³. The data from the Scotian margin cores suggest velocities in a restricted range, 9–16 cm s⁻¹. Maximum velocities for deposition of fine-grained material have been estimated, from laboratory experiments, to be in the range 15–25 cm s⁻¹ (refs 39–41). The present results are therefore in the right range. The very thin silt layers higher in the cores (Fig. 2), should show lower velocities, presumably, but it becomes very difficult to sample these layers to determine the detailed size distribution.

Model for depositional sorting

Within a fine-grained turbidity current there are clay flocs and silt grains having equivalent settling velocities, so that the expected deposit from a waning current would be a graded, silty mud. In most cases, however, the depositional unit is distinctly laminated as well as graded. This separation of mud and silt layers may be primarily due to depositional sorting through the boundary layer at the base of a turbidity current.

The even distribution of grain sizes in the deposits (Fig. 2) suggests an even distribution of grain sizes in the boundary layer, made up of a coarse silt mode and a fine mud tail. The coarser silt settles into the boundary layer as individual grains, while the finer silt and the mud probably settle as flocs where, $D_{mode}(\text{flocs}) > D_{mode}(\text{silt})$, and the effective floc settling velocity is of the same order as that of the modal size of the silt³⁸. The finest mud is left behind in the turbidity current.

As the sediment falls towards the bed the increasing shear in the boundary layer tends to break up the flocs^{39,40} (Fig. 3). The larger silt grains continue to settle through the viscous sublayer to form a silt lamina (Fig. 4a). As more sediment is supplied to the top of the boundary layer the mud concentration increases, and reflocculation may occur (Fig. 4b)^{41,42}. At some critical concentration (Fig. 4c) the clays are able to form aggregates large enough to overcome shear break-up and deposit rapidly through the laminar sublayer as a mud 'blanket' over the coarser silt lamina (Fig. 4d). This must occur rapidly as very little coarse silt is found in the mud layer. The process is then continued with the formation of another silt layer, slightly finer than the first.

Although the separation of mud and silt in this way seems to be a necessary corollary of boundary layer shear, it is not clear exactly what causes the mud to deposit so rapidly. Temporary disturbance of the boundary layer structure (by velocity fluctuations, and so on), or the partial development of a cohesive bed surface as the largest flocs manage to settle through the sublayer, are possible explanations. The increased mud concentration may affect these processes⁴¹ or may be sufficient alone to cause mud blanketing. Experimental work with mud slurries suggests that they deposit more rapidly with increasing concentration⁴².

Discussion

The correlation between silt and mud layers suggests deposition from the same flow, separation arising from a particular value of the bottom stress, τ . If velocity fluctuations were playing a part, silt would presumably be deposited during the strong pulses, and mud during relatively quiet periods. Although this would assist the mud deposition from the boundary layer in the present model, it is not essential to the basic argument. Previous suggestions for the role of velocity fluctuations are in terms of deposition from the flow as a whole, in which case the composition of the whole current must fluctuate with velocity (otherwise the coarser silt would deposit very rapidly during the quieter periods) and no particular correlation would be expected between the size distributions in the mud and silt layers.

In addition, the mud layer would represent low settling velocities and consequently relatively long depositional time scales. The settling velocity of the modal size in the mud layers (Fig. 2) is an order of magnitude less than that of the silt mode. The result would be analogous to that for tidal currents¹³; it takes a long time to deposit a mud layer several mm thick.

The suggestion that lamination is due to a series of thin, turbid flows, one for each lamina^{15,16}, does not explain the overall grading of a unit. However, many laminated, fine-grained sediments do not show any obvious gradation and a more significant objection may be the very long time scales required to deposit a laminated unit, not consistent with the absence of bioturbation.

The possible role of contour currents in the formation of laminated, but ungraded, sediments is harder to assess. Most of the existing literature is concerned either with the reworking of the sediments by steady currents or deposition from slowly moving, nepheloid layers. However, current measurements, close to the sea bed on the upper-continental rise south of Cape Cod, Massachusetts⁴³, have shown that although there is a mean westwards flow along the contours at 5 cm s^{-1} the current pulsates in strength flowing at up to 40 cm s^{-1} to both east and west. The most obvious periodicity in the velocity variations is of the order of 30 d.

If the current varies greatly in strength the resulting sediment dynamics may be similar, except in direction, to those associated with a turbidity current. As far as small scale processes are concerned, a waning contour current would be indistinguishable from a waning turbidity current and the lamination mechanism proposed here would be equally applicable.

Two general comments might be made, the size ranges and concentrations measured in nepheloid layers³⁵ suggest very slow rates of deposition. The importance of winnowing can be assessed directly by comparing the size distribution of a mud layer with that of the silt layer immediately below. A strong correlation between these distributions, as in the data presented here, suggests that material has not been removed by winnowing.

There is, however, direct evidence in the cores that other mechanisms, in addition to shear sorting, are important at some phase of the depositional sequence. In the lowest silt layers in some of the units there is structural evidence for some lamina formation within the graded laminated units by the migration of low-relief bed forms. This probably results from the migration of the coarse silty ripple crest over a muddy trough, and may involve a short amount of tractional transport and sorting. However, this is apparently restricted to the lowest parts of units, and is not seen in the finer silt and clay sizes, consistent with the idea that these sizes do not normally move as bedload.

The primary mechanism for laminae formation seems to be the depositional sorting by the increased shear at the bottom of the bottom boundary layer. The destruction of the flocs leads to differential settling suggested by equation (4) producing a clean, silt layer. The continual accumulation of fine material

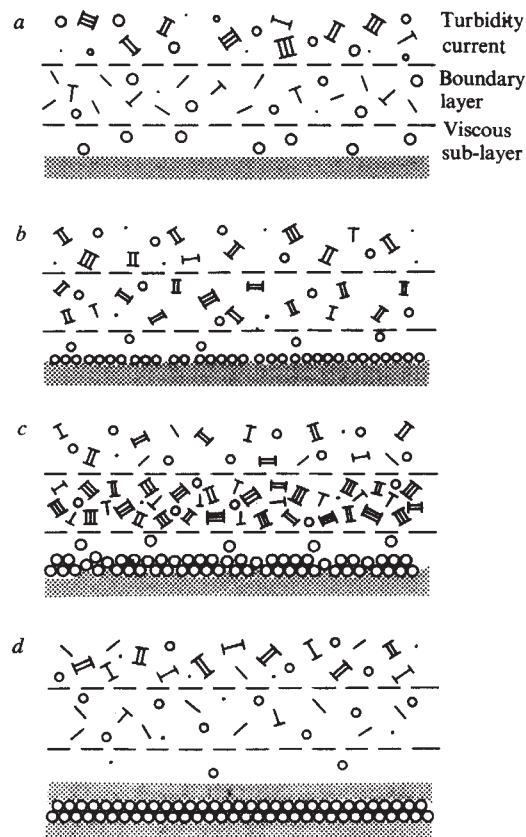
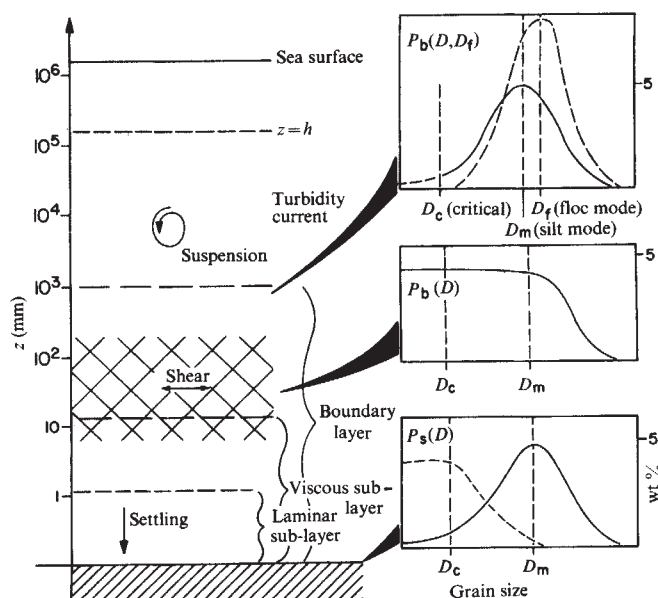


Fig. 4 Schematic representation of silt (○, silt grains various sizes) and mud (I, II, III, clay flocs various sizes) deposition through the boundary layer of a turbidity current. See text for discussion.

Fig. 3. Model for shear sorting in the boundary layer of a turbidity current. Depth measured from sea floor in a mm logarithmic scale. The boxes on the right show the probable grain size distributions at different levels within the boundary layer and the measured grain size distribution of the sediment.



during this phase eventually leads to a rapid deposition of a mud layer; the absence of the coarser silts from this layer indicates that the process is rapid. The rate of deposition of a unit is then primarily controlled by silt deposition whose settling characteristics should be reasonably well described by equation (4). The time for deposition of turbidite units 2–4 cm thick in the Scotian margin cores is therefore estimated to be of the order of 2–4 days. However, to deposit the mud component of the unit at settling velocities appropriate to modal size of the mud would imply time scales of a month or more, even if the bottom stress vanishes.

One of the interesting features of this depositional sorting process is, therefore, the suggestion that the depositional rate from the turbidity current is proportional to the modal size of the silts, the clay flocs falling out being of equivalent settling velocity. The depositional processes as a whole are therefore much more rapid than would be anticipated using any model that views the deposition of the mud layer as a separate process, characterised by the size distribution of the mud alone.

The type of mechanism may well apply to other environments where fine-grained sediments are deposited from slowly moving flows. It should result in the regular silt/mud lamination particularly common in deep sea sediments.

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The natural ovalbumin gene contains seven intervening sequences

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EcoRI fragments of the natural ovalbumin gene were cloned and studied by hybridisation with mature ovalbumin mRNA, electron microscopy, restriction enzyme mapping and limited sequence analysis. The structural gene sequences coding for ovalbumin were found to be separated into eight sequentially orientated pieces by seven intervening sequences of various lengths.

FURTHER insight into the mechanism and regulation of gene expression requires additional knowledge of the molecular organisation of the eukaryotic natural gene. Some information regarding the structure of coding and flanking sequences can be obtained by restriction endonuclease mapping of total genomic DNA followed by hybridisation of the resulting DNA fragments with a probe specific for structural gene sequences¹. Definitive gene structure, however, can only be obtained through molecular cloning of the gene. These two complementary approaches have recently led to the discovery that eukaryotic genes and their mature RNA product may not be strictly colinear, as had been generally assumed². Within the structural gene sequences coding for rabbit and mouse β -globin^{3,4}, mouse immunoglobulin light chain⁵, *Drosophila* ribosomal genes^{6–8} and yeast transfer RNA^{9,10}, regions of DNA sequences have been found which are not represented in the mature RNA product.

Restriction mapping of the ovalbumin gene in chick DNA has previously revealed that the DNA sequences coding for mature ovalbumin mRNA (mRNA_{ov}) are also noncontiguous and are separated by intervening sequences^{11–15}. We report here that successful cloning of the *EcoRI* DNA fragments of the ovalbumin gene has permitted us to construct a detailed

map of the natural gene and compare it to the mature ovalbumin mRNA sequence¹⁶. To our surprise, we have found that the left half of the structural gene is interrupted by a total of seven intervening DNA sequences.

The recombinant plasmid pOV230

A full-length duplex DNA copy of ovalbumin mRNA (cDNA_{ov}) was previously synthesised and cloned¹⁷ in the *Escherichia coli* plasmid pMB9. The ovalbumin DNA in this recombinant plasmid (pOV230) has been sequenced in its entirety¹⁶, and it was shown to contain a copy of all but the terminal 12 base pairs present in the 5'-end of mRNA_{ov}. It contains 64 base pairs of DNA preceding the initiation codon AUG, the entire peptide-coding region for ovalbumin and 634 nucleotide pairs of untranslated sequences following the termination codon UAA. Endonuclease *HhaI* digestion of pOV230 generates a 2.9 kilobase DNA fragment which contains the entire ovalbumin DNA sequence flanked by plasmid DNA. This DNA fragment was separated by agarose gel electrophoresis from the remaining plasmid DNA and used as a specific hybridisation probe for ovalbumin DNA sequences after being labelled with (³²P)dCTP and (³²P)TTP by nick-translation¹⁸ to a specific activity of about 2×10^8 c.p.m. μg^{-1} .

Cloning of the *EcoRI* DNA fragments of the natural ovalbumin gene

All procedures were carried out according to the NIH guidelines in a P3 facility. The *EcoRI* fragments of the ovalbumin gene, partially purified by a combination of RPC-5 column chromatography^{19,20} and preparative agarose gel electro-

phoresis were cloned in the λ gtWES vector^{20,21} as previously described^{22,23}. An *Eco*RI digest of the DNA isolated from one of the recombinant phages generated from hen DNA enriched for the 1.8 kilobase ovalbumin DNA fragment revealed the presence of two chick DNA fragments of 1.8 and 2.0 kilobases (Fig. 1A, lane *a*) but only one of these fragments hybridised with pOV230 (Fig. 1B, lane *a*). These two fragments were subsequently recombined in *E. coli* X1776 after ligation²⁴ with *Eco*RI-digested pBR322 plasmid DNA, and an *Eco*RI digest of the recombinant plasmid (pOV1.8) showed that only the 1.8 kilobase DNA has sequence homology with pOV230 (Fig. 1B, lane *b*). The previously cloned 2.4 kilobase ovalbumin DNA fragment was also recombined in *E. coli* X1776 using the *Eco*RI-digested plasmid vector, pBR322, and an *Eco*RI digest of this recombinant plasmid (pOV2.4) is shown in Fig. 1A and B, lane *c*. The remaining *Eco*RI fragments of the ovalbumin gene were cloned in a similar way and will be reported in detail elsewhere.

Electron microscopy of hybrids formed between the cloned DNA and ovalbumin mRNA

The cloned ovalbumin DNA fragments and purified ovalbumin mRNA were hybridised to allow the formation of R loops, and the hybrids were visualised by electron microscopy. Electron micrographs and line tracings of the hybrid molecules are

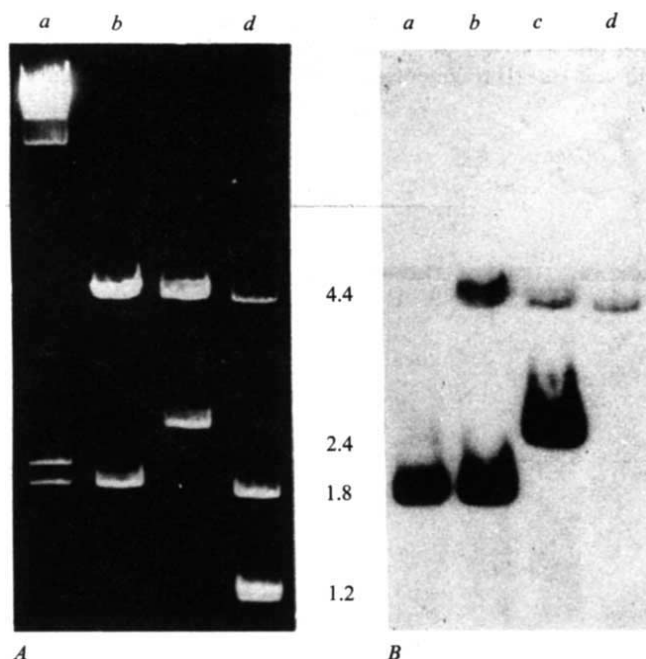


Fig. 1 A, 1% agarose gel electrophoretic separation of the products of digestion of the cloned recombinant DNA with *Eco*RI restriction endonuclease. Electrophoresis was carried out in a Tris-acetate buffer³². *a*, The recombinant λ gtWES DNA, displaying the two cloned DNA fragments of 1.8 and 2.0 kilobases; *b*, The recombinant plasmid pOV1.8 DNA, displaying the cloning vector pBR322 and the cloned 1.8 kilobase ovalbumin DNA; *c*, The recombinant plasmid pOV2.4 DNA showing the cloning vector pBR322 and the cloned 2.4 kilobase ovalbumin DNA; *d*, Molecular weight standards pBR322 *Eco*RI+SV40 *Hind*III of sizes indicated in kilobases. B, hybridisation with (³²P) pOV230 probe of the DNA components displayed in the gel after their transfer to a nitrocellulose paper. As can be seen, only the 1.8 and not the 2.0 kilobase DNA hybridise to an ovalbumin DNA probe. A weaker hybridisation is also observed between pBR322 DNA and the (³²P) pOV230 probe. It represents some residual sequence homology around the *Eco*RI sites of pBR322 and pMB9, the cloning vector of pOV230.

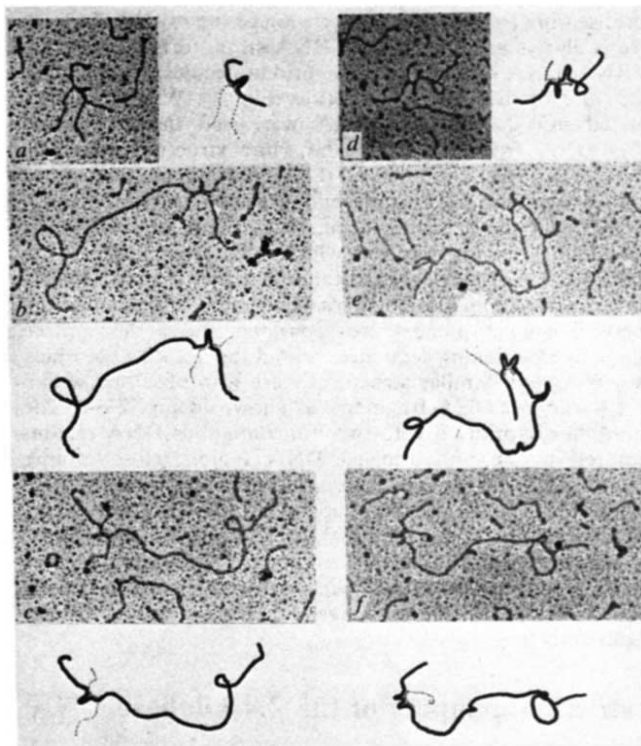


Fig. 2 Electron micrographs of hybrids formed between the cloned DNA and ovalbumin mRNA. Hybrids of the cloned ovalbumin DNA and mRNA_{ov} were formed with 10 μ g per ml of DNA and 20 μ g per ml of purified mRNA_{ov} in 70% deionised formamide containing 100 mM Tris-HCl, pH 7.6, 10 mM Na₂ EDTA and 150 mM NaCl. The mixture was incubated at 42 °C for 15 h. Samples were prepared for electron microscopy as follows: 0.1–0.5 μ g of hybrids in 70% formamide, 0.1 M Tris, pH 8.4, 0.01 M EDTA, 10 μ l of 1 mg per ml cytochrome *c*, were spread onto a hypophase of 40% formamide, 0.01 M Tris, pH 8.4, 0.001 M EDTA. Samples were picked up on collodion-coated 300-mesh copper grids, stained with uranyl acetate, rotary shadowed with platinum-palladium, and examined at 80 keV on a Jeol 100 c electron microscope. *a*, A typical example of a hybrid between pOV1.8 DNA and mRNA_{ov}, the line drawings with the intervening sequence flanked over their side by portions of the structural gene. A solid line is used to represent DNA and a dotted line to represent mRNA_{ov}. *b*, *c*, A similar situation with the 1.8 kilobase piece incorporated into *Bam*HI-cut plasmid DNA. In both examples, portions of the structural gene flank the intervening sequence insert on either side. *d*, An example of a hybrid between the 2.4 kilobase DNA and mRNA_{ov}, with a line drawing interpreting the micrograph. Three pieces of structural gene are indicated, and required to cause the looping out of intervening sequences. *e*, *f*, The same pattern in the 2.4 kilobase DNA incorporated into *Bam*HI-cut plasmid DNA.

shown in Fig. 2. RNA-DNA hybrids were formed in three noncontiguous segments along the 2.4 kilobase ovalbumin DNA, as shown Fig. 2*d*. The displaced single-stranded DNA could not be seen, probably because the R loops occur over short DNA regions and have collapsed. There are two small segments of double-stranded DNA (closed loops) interspersed between the DNA-RNA hybrids because they have no sequence homology with mature ovalbumin mRNA. These DNA segments are intervening sequences which are absent from mature mRNA. Thus, the structural gene sequence within the ovalbumin 2.4 kilobase DNA is subdivided further into at least three noncontiguous segments, separated by DNA sequences of unknown function. The 2.4 kilobase DNA fragment contains only about 450 base pairs of structural gene sequence toward the 5'-end of the gene and is expected to

hybridise only to the 5'-terminal region of the mRNA. Indeed, there is always an unhybridised RNA strand originating from the RNA/DNA junction in the hybrid molecules, representing the 3'-end of the mRNA molecule (Fig. 2). When *Bam*HI-digested pOV2.4 plasmid DNA was used instead of the OV2.4 DNA fragment itself, the same structure could be observed at a specific site about 0.8 kilobases from one end of the linearised recombinant plasmid, as shown in Fig. 2e and f. The fact that the structure occurred in many plasmid DNA molecules at the same location, which is the original *Eco*RI site in pBR322 DNA, strongly indicates that the observed hybrid structure was not an artefact. This experiment suggests that the structural gene sequences are separated into at least three regions by intervening sequences within the 2.4 kilobase chick DNA fragment. Similar structures were also observed within the 1.8 kilobase DNA fragment, as shown in Fig. 2h-c. The mRNA has hybridised with two noncontiguous DNA regions separated by an unhybridised DNA region (closed loop), indicating that the internal structural sequences within the 1.8 kilobase fragment are further subdivided into two portions by an intervening sequence (Fig. 2a). It can also be seen from the electron micrographs that the 1.8 kilobase DNA has sequence homology only to the middle sections of the mRNA, leaving long unhybridised DNA stretches and single-stranded mRNA at both ends (Fig. 2b, c).

Restriction mapping of the 2.4 kilobase DNA fragment

Restriction cleavage sites shown in Fig. 3a are mainly derived from agarose gel electrophoresis of DNA fragments after digestion of the 2.4 kilobase DNA by various restriction enzymes followed by ethidium bromide staining. As shown in Fig. 3a and in close agreement with our previously reported restriction mapping of the natural ovalbumin gene in total chick DNA, the 2.4 kilobase DNA fragment contains a *Pst*I and an *Hind*III cleavage site located 0.42 and 0.25 kilobases, respectively, to the left of the *Eco*RI site that separates the 2.4 and 1.8 kilobase DNA fragments. There is an *Hpa*I and an *Hae*III cleavage site 0.17 and 0.6 kilobases from the other *Eco*RI terminus because an *Hpa*I or *Hae*III digestion of the 2.4 kilobase fragment resulted in two fragments of 0.17 and 2.23 kilobases, or 0.6 and 1.8 kilobases, respectively, with the latter fragments susceptible to *Pst*I and *Hind*III digestion (data not shown).

An *Sst*I digestion of the 2.4 kilobase fragment has resulted in a 1.6 kilobase and two 0.4 kilobase DNA fragments. One of the two 0.4 kilobase *Sst*I fragments can also be obtained from digesting the 0.6 kilobase *Hae*III fragment (data not shown). Accordingly, there should be an *Sst*I site 0.2 kilobases to the left of the *Hae*III site (Fig. 3a). The other 0.4 kilobase *Sst*I fragment was digested by *Hind*III to two fragments of 0.1 and 0.3 kilobases, but was resistant to *Pst*I digestion. The second *Sst*I site must, therefore, be located between the *Pst*I and the *Hind*III sites, and is very close to the right of the *Pst*I site, as the 0.4 kilobase *Pst*I fragment was only slightly larger than the *Sst*I fragment.

Four DNA fragments result from *Hinf*I digestion (Fig. 4a), indicating the presence of three *Hinf*I cleavage sites in the 2.4 kilobase DNA. The two *Hinf*I fragments derived from the left and right ends were identified as the 0.75 and 1.5 kilobase pieces by secondary restriction digests using *Hae*III or *Hind*III, respectively (Fig. 4a). There are a total of seven DNA fragments after *Mbo*II digestion of the 2.4 kilobase DNA (Fig. 4a), indicating the presence of six *Mbo*II sites. The terminal *Mbo*II sites were determined by identifying the *Mbo*II fragments that were susceptible to digestion by *Hind*III and *Hpa*I (Fig. 4a). The locations of the remaining *Mbo*II sites were determined from a partial *Mbo*II endonuclease digest. The 2.4 kilobase fragment was labelled at the 5'-terminus using T4 polynucleotide kinase in the presence of γ (³²P)ATP. The end-

labelled product was digested with *Hind*III to generate two radioactive fragments of 2.1 and 0.25 kilobases that were separated by preparative gel electrophoresis. The labelled fragments were digested with *Mbo*II and aliquots (~10,000 c.p.m.) were taken at various times and subjected to electrophoresis followed by autoradiography. The 0.25 kilobase fragment was apparently not digested by *Mbo*II. A total of six additional radioactive bands of different sizes were obtained from the 2.1 kilobase fragment (Fig. 5A). The concomitant decrease and increase in the intensities of the larger and smaller radioactive bands, respectively, with the digestion time confirm that there are a total of six *Mbo*II sites within the 2.4 kilobase fragment (Fig. 5A). From the sizes of the various partial digestion products, the *Mbo*II sites can be estimated to be 0.42, 1.0, 1.2, 1.4, 1.5 and 1.7 kilobases to the right of the *Eco*RI site on the left hand end of the 2.4 kilobase fragment (Fig. 3a). The locations of the terminal *Mbo*II sites therefore agree with the data shown in Fig. 4a. Similarly, three *Hinf*I sites were found to be located 0.75, 1.0 and 1.1 kilobases to the right of the left *Eco*RI site on the 2.4 kilobase ovalbumin DNA fragment (Fig. 3a), in agreement with the data shown in Fig. 4a.

Localisation of structural gene regions within the 2.4 kilobase DNA

The 2.4 kilobase DNA fragment was next digested with a variety of restriction endonucleases and the fragments were assayed for the presence of structural ovalbumin sequences by hybridisation with (³²P)pOV230 and autoradiography after electrophoresis on an agarose gel²⁵. Both the 0.6 and 1.8 kilobase *Hae*III fragments yielded strong hybridisation signals,

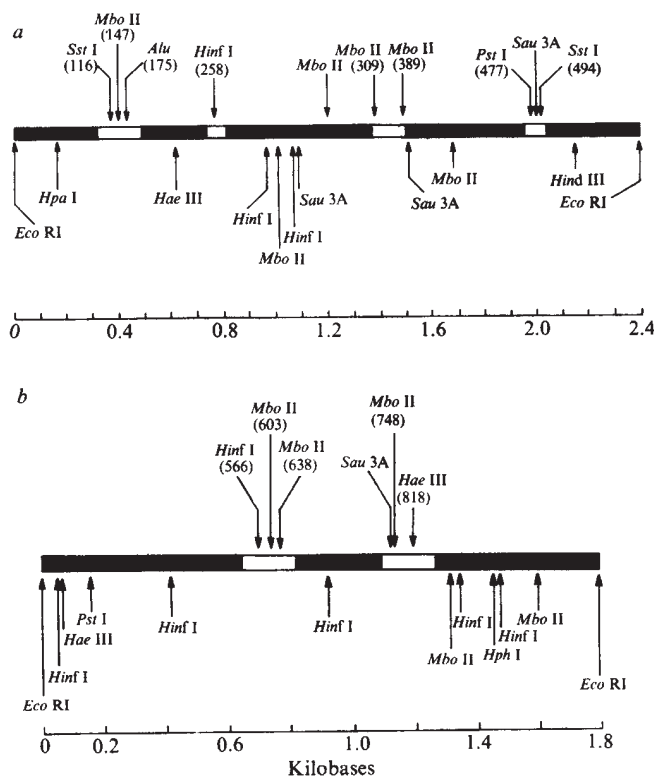


Fig. 3 Restriction endonuclease maps of the cloned 2.4 kilobase (a) and the 1.8 kilobase (b) ovalbumin DNA. The endonuclease restriction sites are indicated by arrows. Numbers in parentheses refer to the corresponding sequences in ovalbumin mRNA¹⁶. Black sections on the map represent intervening DNA sequences, white sections represent ovalbumin structural DNA.

indicating the presence of substantial portions of the structural gene sequence in both fragments (Fig. 5B). In the case of *Pst*I digestion, only the 2.0 kilobase fragment yielded a strong hybridisation signal (data not shown). With prolonged

autoradiography, however, the 0.42 kilobase fragment also yielded a very weak band, indicating the presence of little structural gene sequence within the 0.4 kilobase fragment. We conclude that some of the structural gene sequence must be located between the *Pst*I and *Hind*III sites, as the 0.25 kilobase *Hind*III fragment failed to hybridise with pOV230. Furthermore, the 0.76 kilobase *Mbo*II fragment containing the *Pst*I and *Hind*III sites hybridised strongly with pOV230 (Fig. 5B). Thus, our data suggested that the *Pst*I site in the 2.4 kilobase fragment could be the *Pst*I site 477 base pairs from the 5'-end of the structural ovalbumin gene (pOV230) (ref. 16). Indeed, an *Sau*3A site was found between the *Pst*I and the neighbouring *Sst*I site in the 2.4 kilobase fragment (data not shown); this resembles a cluster of cleavage sites consisting of *Pst*I (477), *Sau*3A (484) and *Sst*I (492) in the structural gene.

An 80 base pair *Mbo*II fragment showed a weak hybridisation band with pOV230 on prolonged autoradiography, so that it may have been derived from the *Mbo*II sites at positions 308 and 389 in the structural gene. The hybridisation signal was weak, mainly because the small DNA fragment became diffuse in the agarose gel. Indeed, there is an *Hph*I site within the 80 base pair *Mbo*II fragment in the 2.4 kilobase DNA which must be the *Hph*I site at position 336 within the structural gene.

The 0.42 kilobase *Mbo*II fragment from the left hand end of the 2.4 kilobase fragment hybridised with pOV230 (Fig. 5B). The *Sst*I site immediately to the left of the *Mbo*II sites resembles those present in the 5'-end of the structural gene at positions 116 and 147, respectively. Furthermore, this structural gene region must be terminated before reaching the *Hinf*I (258) site in the structural gene, as the nearest *Hinf*I site in the 2.4 kilobase fragment is more than 300 base pairs away.

Although the observation of three noncontiguous structural gene regions within the 2.4 kilobase fragment would have confirmed the presence of two intervening sequences, as indicated by the electron micrographs shown in Fig. 2, it does not fully account for all the restriction mapping and hybridisation data. As there is no *Hinf*I site located within 50 base pairs to the left of the 80 base pair *Mbo*II fragment, the *Hinf*I (258) and *Mbo*II (308) sites in the structural gene (pOV230) must be separated in the natural gene. As the *Hinf*I (258) site is not contiguous with both the structural gene regions containing the *Sst*I (116) and the *Mbo*II (308) sites, it should be present in a fourth structural gene region within the 2.4 kilobase DNA. Indeed, the *Hinf*I site 0.15 kilobases to the right of the single *Hae*III site in the 2.4 kilobase fragment is the *Hinf*I (258) site in the structural gene, as DNA sequences around this *Hinf*I site were found to be identical to the sequences flanking the *Hinf*I (258) site by direct sequence analysis.

Thus, the structural gene sequences within the 2.4 kilobase DNA were further subdivided into four portions by three intervening sequences, as shown in Fig. 3a. In retrospect, we observed only two intervening sequences in the electron micrograph (Fig. 2) because the second structural gene portion containing the *Hinf*I (258) site was probably too short to form a stable hybrid with the mRNA in the conditions used.

Restriction mapping of the 1.8 kilobase DNA

Most of the data required to map the 1.8 kilobase DNA fragment shown in Fig. 3b are again derived from gel separation of DNA fragments from various restriction digests of the 1.8 kilobase DNA (Fig. 4b-d). The positions of the unique *Pst*I site 0.15 kilobases from the left hand end and the *Hae*III site 0.57 kilobases from the right hand end are consistent with our previous data obtained by mapping chick DNA¹³. There is an *Hph*I site 0.35 kilobases from the right hand end because it cleaves either the 1.8 kilobase DNA or the 0.57 kilobase *Hae*III fragment into pieces of 0.35 and 1.45 kilobases or 0.35 and 0.22 kilobases, respectively (Fig. 4b and c). *Sau*3A

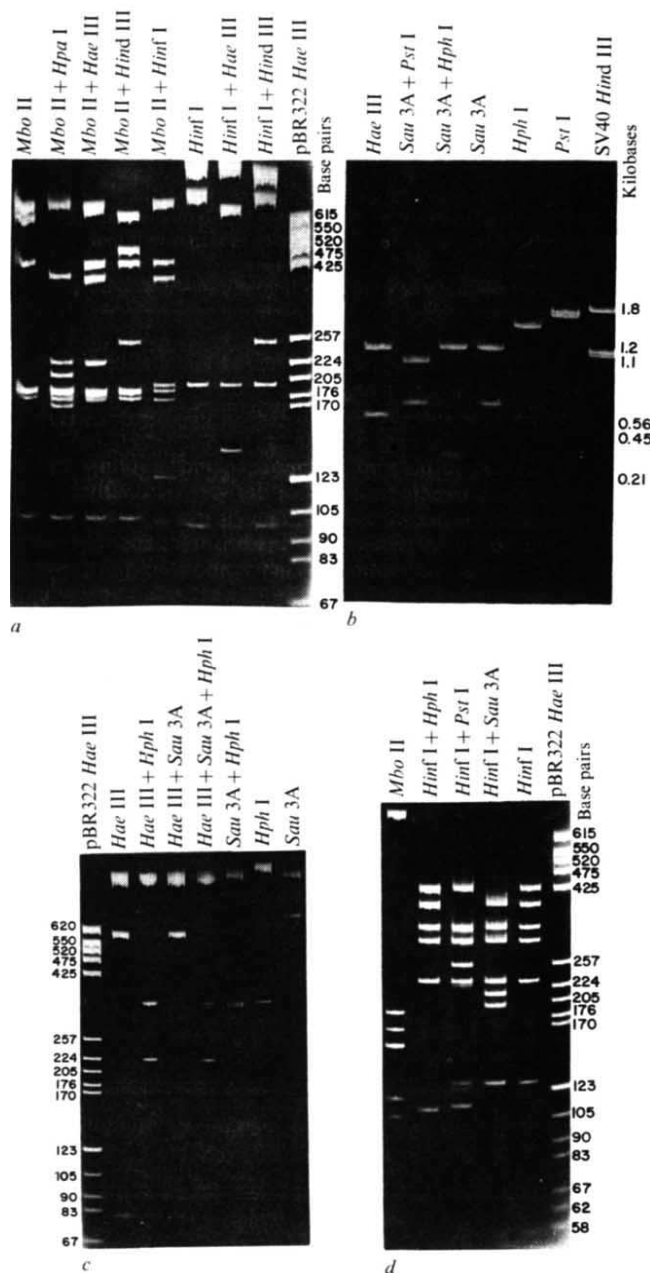


Fig. 4 Gel electrophoretic separation of the products of digestion of the cloned DNA with various restriction endonucleases. Restriction enzymes are listed above each lane, which displays the resulting DNA fragments. Molecular weight standards are separated in the last lane of each gel and their sizes in base pairs or kilobases are as indicated. *a*, A 5% acrylamide gel separation in Tris-borate buffer³³ of the digestion products of the 2.4 kilobase DNA. The *Hpa*I endonuclease used in the *Mbo*II+*Hpa*I digestion was contaminated with *Hpa*II activity which cleaved the *Mbo*II 0.62 kilobase DNA fragment into the 0.4+0.22 kilobase DNAs. The cleavage site for *Hpa*I is within the 0.4 kilobase DNA, which is cleaved into the 0.21+0.17 kilobase fragments. *b*, A 1.2% agarose gel separation in Tris-acetate buffer³² of the digestion products of the 1.8 kilobase DNA. *c*, *d*, 5% acrylamide gel separation in Tris-borate buffer³³ of the digestion products of the 1.8 kilobase DNA.

cleaves the 1.8 kilobase DNA into two fragments of 1.15 and 0.65 kilobases, and the latter fragment is in turn cleaved by *Hae*III into two fragments of 0.57 and 0.07 kilobases (Fig. 4b and c). A *Sau*3A site must therefore be present about 0.07 kilobases to the left of that *Hae*III site. *Hinf*I and *Mbo*II again digested the 1.8 kilobase DNA into seven and eight DNA fragments, respectively (Fig. 4d), and their respective cleavage sites were again ordered from the termini by partial digestion of the end-labelled 1.8 kilobase DNA followed by autoradiography (not shown). Digestion of the *Hinf*I fragments by *Sau*3A, *Pst*I of *Hph*I showed that the 0.42, 0.4 and 0.12 *Hinf*I fragments were each cleaved into smaller fragments (Fig. 4d), confirming the localisation of these sites from the end-labelling experiment.

Localisation of structural gene regions within the 1.8 kilobase DNA

The 1.8 kilobase DNA fragment was digested with several restriction endonucleases and the resulting DNA fragments were assayed for the presence of structural ovalbumin sequences by hybridisation with (32 P)pOV230 and autoradiography after electrophoresis on agarose gels. Little, if any, structural ovalbumin gene sequence can be located to the right of the right hand *Hae*III site (Fig. 3b) because the 0.57 kilobase *Hae*III fragment failed to hybridise with pOV230 and only the 1.1 kilobase fragment showed a strong hybridisation signal (Fig. 5C). On the other hand, both the 1.15 and 0.65 kilobase DNA fragments resulting from a *Sau*3A digestion of the 1.8 kilobase DNA showed strong hybridisation signals with pOV230 (Fig. 5C). The presence of a third hybridising component at 1.8 kilobases was due to incomplete digestion of the DNA by *Sst*I. Thus, some structural gene sequence must be present between the unique *Sau*3A site and the right hand *Hae*III site. The organisation of *Sau*3A and *Hae*III sites in the 1.8 kilobase fragment also resembles that of *Sau*3A (751) and *Hae*III (818) in the structural gene. Furthermore, a third *Mbo*II site is present in the same region which may represent the *Mbo*II (748) site in the structural gene. Indeed, the 0.43

kilobase *Hinf*I fragment containing all these sites hybridised strongly with pOV230 (Fig. 5C). A second cluster of restriction sites present in the 1.8 kilobase DNA is the *Hinf*I and two successive *Mbo*II sites about 0.7 kilobases from the left hand end; this resembles the *Hinf*I (566), *Mbo*II (603) and *Mbo*II (638) in the structural gene (Fig. 3b). This is confirmed by the observation that the 0.23 kilobase *Hinf*I fragment containing these sites did hybridise with pOV230 (Fig. 5C). However, the two clusters of restriction sites, *Hinf*I (566)–*Mbo*II(603)–*Mbo*II (638) and *Mbo*II (748)–*Sau*3A (751)–*Hae*III (818) are separated farther by 0.25 kilobases on the cloned natural ovalbumin DNA than the same two restriction clusters on pOV230, indicating that there must be an intervening DNA sequence between them in the natural ovalbumin gene. This agrees closely with our electron microscopic results (Fig. 2). Evidence for the existence of multiple intervening sequences in the ovalbumin gene has also been obtained by Garapin *et al.*²⁶.

A search for the 5'-end of the ovalbumin gene

A restriction site close to the 5'-end of the structural gene (pOV230) that has been identified in the 2.4 kilobase DNA fragment is the *Sst*I (116) site. It is located about 0.4 kilobases from the left hand terminus of the 2.4 kilobase fragment, and there is sufficient DNA sequence remaining in the fragment to accommodate the remaining 5'-end of the gene. In the structural ovalbumin gene, however, there is yet another restriction endonuclease site, *Taq*I, that cleaves the gene at position 41. Indeed, the left hand end of pOV230 can be cleaved by this enzyme¹⁶. However, the 0.4 kilobase *Sst*I fragment was not cleaved by it. We conclude that the 5'-terminus of the ovalbumin gene is not located within the 2.4 kilobase DNA fragment. We have confirmed this by direct sequence analysis.

Prolonged autoradiography of total *Eco*RI-digested chick DNA after hybridisation with labelled pOV230 has revealed the presence of a weak band, tentatively sized at 4.2 kilobases, which may contain the 5'-terminus of the ovalbumin gene. We have now cloned this DNA fragment.

The 3'-end of the ovalbumin gene

We have also cloned the 9.5 kilobase DNA fragment which contains the 3'-end of the ovalbumin gene and have not found any evidence that the structural sequence may be further divided by intervening sequences. The organisation of structural gene sequences within this fragment is in excellent agreement with our previous determinations¹³. Of particular interest is the observation that the extensive 634 nucleotide pairs of untranslated region following the termination codon UAA in the structural gene contains no intervening sequences. Thus, the fact that all seven intervening sequences occur within the left hand half of the sequences coding for mRNA_{ov} demonstrates a striking nonrandom distribution.

Significance of the 1.3 kilobase ovalbumin DNA

It has previously been reported that the 1.3 kilobase *Eco*RI fragment may be allelic with the 1.8 kilobase DNA fragment¹⁵. This hypothesis can now be tested directly using nick-translated (32 P)OV1.8 cloned DNA to hybridise with total *Eco*RI-digested chick DNA. As both the 2.4 and 1.8 kilobase fragments are unique chick DNA sequences¹⁴, they are expected to hybridise only with chick DNA fragments of 2.4 and 1.8 kilobases, respectively. When the (32 P) 2.4 kilobase DNA probe was hybridised to total chick DNA digested with *Eco*RI, it

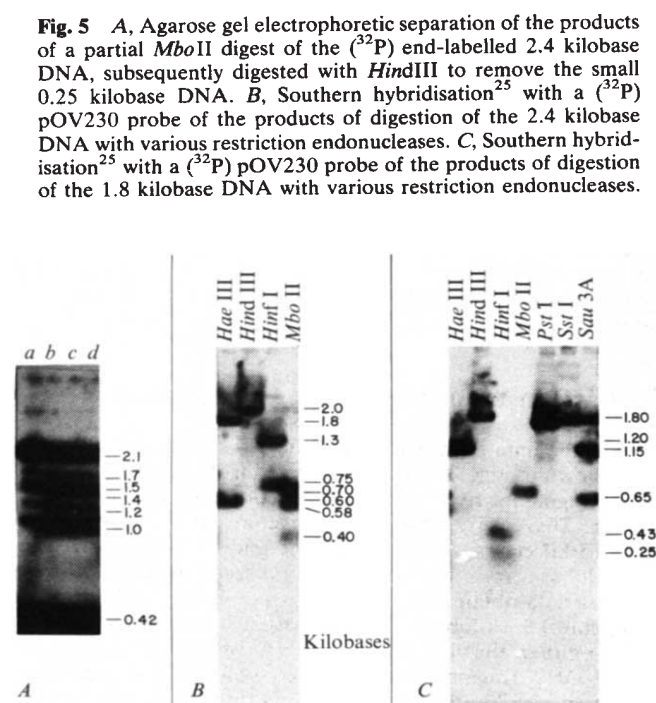


Fig. 5 A, Agarose gel electrophoretic separation of the products of a partial *Mbo*II digest of the (32 P) end-labelled 2.4 kilobase DNA, subsequently digested with *Hind*III to remove the small 0.25 kilobase DNA. B, Southern hybridisation²⁵ with a (32 P) pOV230 probe of the products of digestion of the 2.4 kilobase DNA with various restriction endonucleases. C, Southern hybridisation²⁵ with a (32 P) pOV230 probe of the products of digestion of the 1.8 kilobase DNA with various restriction endonucleases.

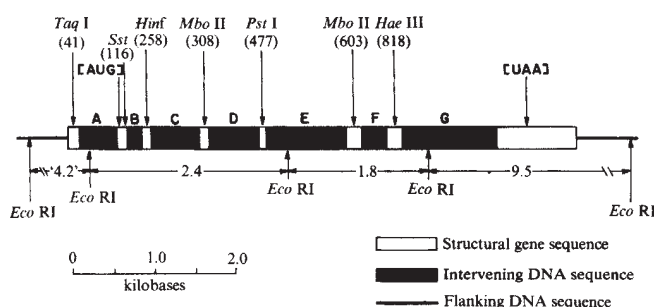


Fig. 6 A map of the organisation of structural and intervening DNA sequences comprising the chick ovalbumin gene. Each of the noncontinuous structural segments is identified by one restriction endonuclease site. The positions of the initiation and termination codons are taken from L. McR. *et al.*¹⁶.

generated only a 2.4 kilobase band. Hybridisation with the 1.8 kilobase DNA probe, however, generated two bands at 1.8 and 1.3 kilobases (E.C.L. *et al.*, manuscript in preparation), suggesting that the 1.3 and 1.8 kilobase *Eco*RI DNA fragments represent allelic ovalbumin DNA in the chick genome. These results agree with the conclusions of Weinstock *et al.*¹⁵ emanating from studies of chickens of differing genetic strains. In fact, we suggest that this allelic (1.8 compared with 1.3) combination may represent only one of a newly emerging series of genetic alleles which have no phenotypic representation, as mutations may occur only in the intervening sequences of genes rather than the peptide-coding region. Thus, such alleles can be demonstrated definitively only by detailed analysis of cloned intervening regions of the natural genes, but not by analyses of the sequences of mRNA, cDNA or the proteins themselves.

Comments on the sequence organisation of the ovalbumin gene

The structure and sequence organisation of the natural ovalbumin gene is shown in Fig. 6. The order and orientation of the 2.4, 1.8 and 9.5 kilobase ovalbumin DNA-containing fragments were established previously by restriction mapping of genomic chick DNA. The '4.2' kilobase DNA is tentatively placed at the missing 5'-end, although the structural gene sequence of the 5'-terminal nucleotide pairs has not yet been identified within this DNA fragment. The comparison of restriction cleavage sites and limited DNA sequence determinations of the cloned DNA fragments with the structural gene sequence have demonstrated that the latter is divided into eight portions in genomic chick DNA by seven intervening DNA sequences with unknown functions. It is possible that this gene is even further subdivided into very small alternating regions of structural and intervening DNA sequences that are not detectable in the present analysis. This possibility can only be eliminated by chemical sequence analysis of the entire natural gene.

Despite the intricate structure of the natural ovalbumin gene, a correct structural sequence may be obtained from left to right by omitting the intervening sequences. We have previously reported that the intervening sequences within the ovalbumin gene are unique chick DNA sequences and are transcribed in their entirety during gene expression²³. Furthermore, the expression of the intervening sequences is inducible by steroid hormones in a coordinate manner with the structural gene sequences. This observation raises the possibility that the entire ovalbumin gene is transcribed into a precursor RNA which is at least three times the size of the mature messenger RNA, and that the intervening sequences are subsequently enzymatically processed away from the

mature mRNA by precise excision and proper ligation. Indeed, our preliminary experiments have indicated the presence of discrete nuclear RNA bands up to 40S in size which are capable of hybridising with pOV230, pOV2.4 and pOV1.8 after electrophoresis in denaturing gels. These observations, with our new appreciation of eukaryotic gene structure, should greatly facilitate our efforts to understand steroid hormone action in the chick oviduct²⁷⁻³¹.

The observation that all but one of the intervening sequences are present within the peptide-coding portion of the gene is noteworthy. The remaining intervening sequence occurs before the initiation codon AUG and there is no intervening sequence within the 634 nucleotide pairs of the untranslated region toward the 3'-terminus of the gene. The distribution of intervening sequences among structural gene regions is therefore nonrandom. The exact significance of the uneven distribution may only be determined after the biological function(s) of the intervening sequences is established.

Of further interest in the case of the ovalbumin gene is that over 7,000 base pairs of DNA sequence are required to code for a mature mRNA of 1,859 bases. This observation raises the question as to whether there is really so much excess or superfluous DNA in the eukaryotic genome as has been previously thought.

Finally, the existence of intervening sequences within structural genes was unpredicted by our prior understanding of eukaryotic genes. They introduce a new complexity into genetic analysis because sequence differences within intervening sequences can create allelic genotypes which have no phenotypic manifestations and thus escape present methods of analysis. Their existence can be revealed only by molecular cloning and sequence analysis of the natural gene.

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Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950

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Comparison of the oligonucleotide maps of the RNAs of current human influenza (H1N1) virus isolates shows these strains to be much more closely related to viruses isolated in 1950 than to strains which circulated before or after that period.

IN May 1977 influenza viruses of the H1N1 (haemagglutinin and neuraminidase) serotype were isolated in China¹. Subsequently, additional H1N1 virus isolates were obtained in

Russia and in the winter of 1977–78 epidemic infection with these H1N1 ('Russian') viruses was evident all over the Northern Hemisphere, including the United States^{2,3}. Although investigators had speculated that the H3N2 subtype which first appeared in 1968 was approaching the end of its period of prevalence, the emergence of H1N1 virus was unexpected. H1N1 viruses were epidemic between 1946 and 1957 and a large proportion of the world's population over the age of 20 possesses serum antibodies against such viruses. Consequently, a strain with a novel haemagglutinin or one which had appeared less recently would have seemed a more likely candidate for the next pandemic strain. Because of the antigenic relatedness of the surface proteins of the 1946–57 H1N1 strains and the Russian isolates we set out to compare the genomes of these viruses by oligonucleotide mapping. Such

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Table 1 Summary of the comparison of oligonucleotide patterns derived from the RNAs of different influenza viruses

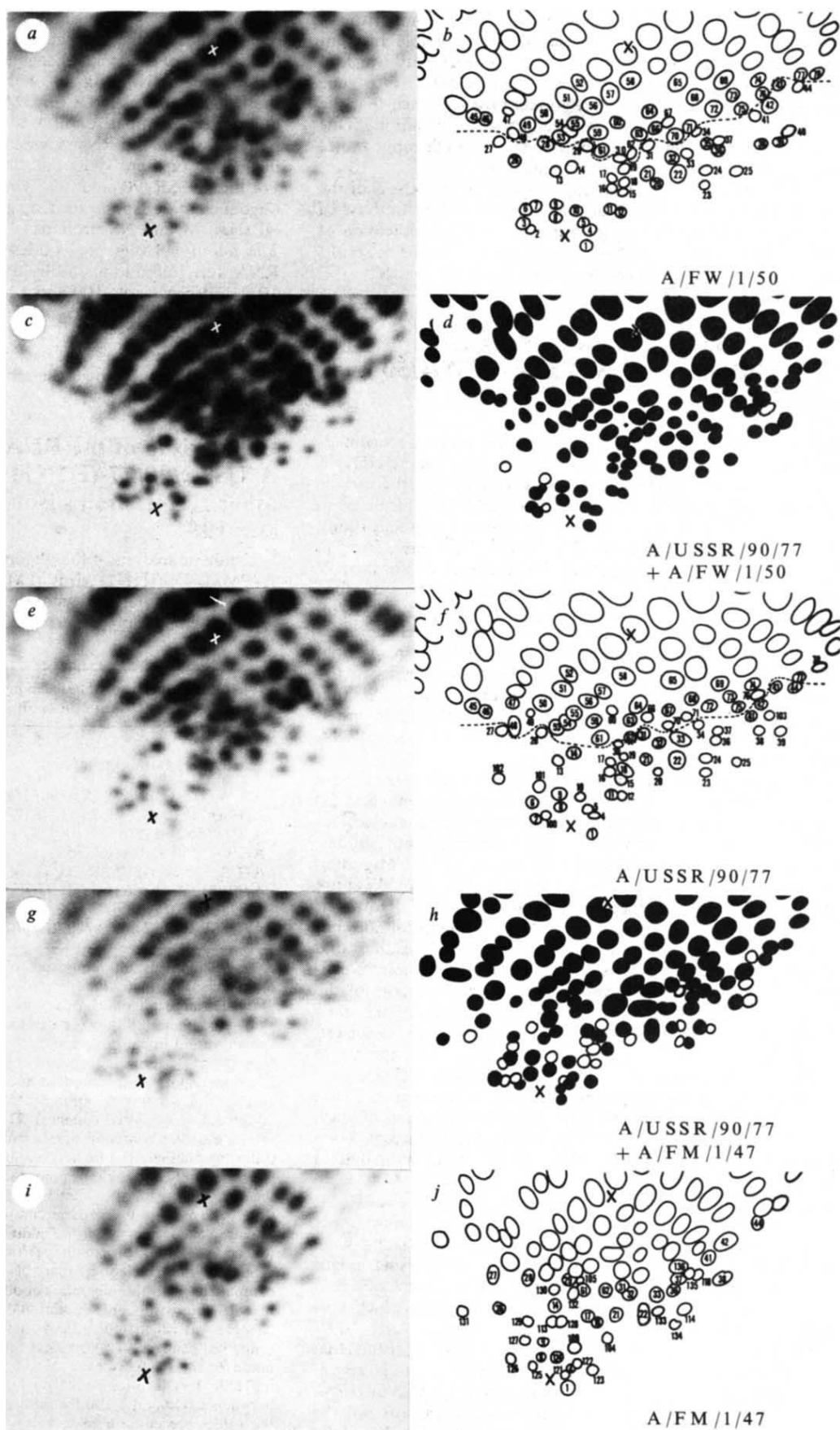
Strain	Spots missing*	Spots additional*	Total no. of spots evaluated†
A/FW/1/50 (H1N1)	—		44
A/USSR/90/77(H1N1)	3, 7, 26, 29, 35, 40	100, 101, 102, 103	42
A/USSR/92/77(H1N1)	3, 5, 7, 10, 12, 15, 26, 29, 30, 35, 40	100, 101, 102, 103, 104, 105	39
A/HK/123/77(H1N1)	5, 6, 7, 15, 16, 26, 29, 35, 40	101, 102, 103, 104, 105, 106, 107	42
A/I/2/50(H1N1)	5, 15, 26, 40	107, 108	42
A/C/1/56(H1N1)	1, 4, 6, 7, 8, 10, 11, 12, 15, 19, 21, 23, 24, 27, 29, 31, 33, 35, 39, 40	102, 103, 105, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120	37
A/FM/1/47(H1N1)	2, 3, 5, 6, 7, 10, 11, 12, 13, 15, 16, 19, 20, 23, 24, 25, 34, 35, 39, 40, 43	104, 105, 109, 113, 114, 118, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136	44
A/PR/8/35(H0N1)	2, 4, 5, 8, 9, 11, 12, 15, 16, 20, 22, 23, 24, 25, 27, 35, 40, 44	102, 106, 113, 114, 118, 119, 123, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147	45
A/PtCh/1/73(H3N2)	2, 3, 4, 7, 8, 9, 13, 15, 16, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 33, 35, 38, 40, 44	101, 102, 103, 113, 116, 127, 130, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166	46

The WHO system of nomenclature²⁴ is used here: this designates influenza viruses according to: type/place of isolation/isolate number/year of isolation (subtypes of haemagglutinin, H, and neuraminidase, N). The abbreviations FW, USSR, HK, I, C, FM, PR, PtCh refer to the following locations: Fort Warren, Wyoming; Soviet Union, place unknown; Hong Kong; Irvington House, New York; Cornell University, New York; Fort Monmouth, New Jersey; Puerto Rico; Port Chalmers, New Zealand.

* The ribonuclease T₁-resistant oligonucleotides of the RNA of influenza A/FW/1/50 virus were separated by two-dimensional gel electrophoresis (Fig. 1a) and the large oligonucleotides were numbered 1–44. By coelectrophoresis of the oligonucleotides of the RNAs of FW virus and of other strains (for example, Fig. 1c, g) it was determined which FW virus-specific spots were missing in a particular pattern (spots missing). Similarly, spots were identified which were absent in the FW virus pattern and present in patterns of the other viruses (additional spots). These spots were labelled 100–162. Oligonucleotides not distinguishable from those present in the FW virus pattern retained the FW virus oligonucleotides designation (1–44). Oligonucleotides present in more than one virus (other than the FW virus) retained the original designation (100–162).

† The oligonucleotide spots of the viruses used in this analysis are identified in Figs 1, 2, 3.

Fig. 1 Oligonucleotide maps of the RNAs of different H1N1 influenza viruses. *a*, RNA of influenza A/FW/1/50 virus. *b*, Diagram of (*a*). *c*, Mixture of the RNAs of influenza A/FW/1/50 and A/USSR/90/77 viruses. *d*, Diagram of (*c*). *e*, RNA of influenza A/USSR/90/77 virus. *f*, Diagram of (*e*). *g*, Mixture of the RNAs of influenza A/USSR/90/77 and A/FM/1/47 viruses. *h*, Diagram of (*g*). *i*, RNA of influenza A/FM/1/47 virus. *j*, Diagram of (*i*). All influenza virus strains were replicated in embryonated eggs. Purified virus was subsequently phenol-extracted^{17,18} and the RNA (0.5 µg) was digested with ribonuclease T₁¹⁹. The resulting oligonucleotides were 5'-end labelled with [γ -³²P]ATP using γ -poly-nucleotide kinase¹⁹ and separated by two-dimensional polyacrylamide gel electrophoresis (refs 20–23 and W. A. Haseltine, personal communication). The first dimension (left to right) was at pH 3.5 on a 10% polyacrylamide gel, and the second dimension (bottom to top) was at pH 8 in a 21.8% polyacrylamide gel. The large oligonucleotide spots of the RNA of influenza A/FW/1/50 virus are numbered 1–78 (diagram *b*). In patterns of other viral RNAs, those oligonucleotide spots which migrate indistinguishably from spots of FW virus RNA retain the FW virus number. Additional spots (migrating differently from those in the FW virus pattern) are given numbers above 100 (diagram *f*, *j*). For comparison of the RNAs of A/FW/1/50 and A/USSR/90 viruses approximately 80 spots were used because fingerprint maps of the isolated genes were available. In order to compare the oligonucleotide patterns of the total RNAs of all influenza viruses analysed in this study we used only ~40 oligonucleotides in each case (see text and Table 1) (spots below the broken lines in diagrams *b* and *f*). In diagram *d* the black spots (full circles) indicate the oligonucleotides of the A/FW/1/50 virus RNA. The open circles represent the additional spots of the RNA of A/USSR/90 virus. Similarly in diagram *h*, open circles represent the additional A/USSR/90 virus-specific spots and the A/FM/1/47 specific spots are shown as full circles. The positions of the dye markers, xylene cyanole FF and bromophenol blue, are indicated by crosses.



In diagram *d* the black spots (full circles) indicate the oligonucleotides of the A/FW/1/50 virus RNA. The open circles represent the additional spots of the RNA of A/USSR/90 virus. Similarly in diagram *h*, open circles represent the additional A/USSR/90 virus-specific spots and the A/FM/1/47 specific spots are shown as full circles. The positions of the dye markers, xylene cyanole FF and bromophenol blue, are indicated by crosses.

analysis is a more comprehensive method for the comparison of the genetic relatedness of viruses than is possible by conventional measurements of serologic cross-reactivity which in the case of influenza viruses is mediated by only 2 of 8 gene products. In addition, we were looking for evidence that the Russian strains may have emerged by recombination. (One mechanism which has been proposed to explain the appearance of new strains involves recombination⁴⁻⁸.)

Analysis of the oligonucleotide patterns of the RNAs of the Russian viruses revealed that they are very similar to those of strains isolated in 1950 and that there was no indication of recombination in the origin of the Russian viruses. The results leading to these conclusions are described in this article.

Comparison of RNAs of influenza A/USSR/90/77 (H1N1) and A/FW/1/50 (H1N1) viruses

Serologic examination had revealed that both the haemagglutinin (HA) and the neuraminidase (NA) of the new H1N1 (Russian) isolates were antigenically very similar to the surface glycoproteins of the A/FW/1/50 strain (FW virus) isolated in 1950 (ref. 2 and A. P. Kendal, personal communication). Therefore, we compared the RNA of one Russian (A/USSR/90/77) isolate (USSR/90 virus) with that of the FW virus. Figure 1 shows the two-dimensional patterns of ribonuclease T₁-resistant oligonucleotides of FW and USSR/90 virus RNAs (Fig. 1a, b, e, f). Close examination of the maps revealed many similarities between the two RNAs. In addition, analysis of the pattern obtained by coelectrophoresis of the oligonucleotides of both viral RNAs revealed (Fig. 1c, d) that only four spots (white circles) of the USSR/90 virus migrated differently from corresponding spots of the FW virus RNA (dark circles) (see also Table 1).

In order to estimate the extent of variation between these two viral RNAs the following assumptions have been made. (1) Although large ribonuclease T₁-resistant oligonucleotides make up only a fraction of the total number of nucleotides, they are representative of the entire genome. (2) Migration differences of oligonucleotides in the two-dimensional gel are associated with differences in primary structure. (However, 'common' spots indistinguishable by gel migration do not necessarily possess identical sequences, suggesting that differences detected by this method represent a minimal estimate of the extent of variation.) (3) Differences in the oligonucleotide patterns of closely related RNAs are most likely the result of single base changes (for comparisons see ref. 9). Such a conclusion is probably incorrect when applied to oligonucleotide patterns of more distantly related RNAs.

Based on these assumptions and the data included in Fig. 1 and Table 1 we estimated the minimum number of base changes between the RNAs of FW and USSR/90 viruses. For a comparative analysis of the RNA of FW virus with that of USSR/90 and other viruses, we used only 44 large oligonucleotides (numbered 1-44 in Fig. 1b) of FW virus RNA. This number although arbitrary was chosen because these oligonucleotides were most readily distinguishable and were most likely to represent unique sequences. Using oligonucleotide markers²⁰ we found that the 44 oligonucleotides of FW virus represent a total of 1,077 nucleotides which corresponds to 7.6% of the total genome (molecular weight 4.9×10^6) (ref. 10). Calculations then show (see Table 2 legend) that the minimum base sequence difference between the large oligonucleotides of FW and USSR/90 virus is 8/1,088 or 0.7%.

Our next concern was to determine whether or not the four 'additional' spots (100, 101, 102 and 103 in Fig. 1d) of the USSR/90 virus RNA were associated with a single gene. The RNAs of USSR/90 and FW viruses were separated on polyacrylamide gels as previously described^{19,20}. The three *P* genes together, the *HA* genes, the *NP* and *NA* genes together, the *M*

genes and the *NS* genes were eluted from the gel^{20,21} and oligonucleotide maps of the isolated genes were obtained. In the analysis of the isolated segments we compared 78 spots of the FW virus RNA (Fig. 1b) with a similar number of spots of the USSR/90 virus RNA (Fig. 1f). This number is higher than that used for comparison of total RNAs (44 spots) because the patterns of isolated RNA segments are simpler and permit the distinction of more spots than the maps of total viral RNAs. For the USSR/90 and FW viruses, we assigned individual oligonucleotide spots to the genes coding for *P*₁₋₃, *HA*, *NP/NA*, *M* and *NS* proteins, respectively (data not shown). The additional oligonucleotides 100-103 of USSR/90 virus RNA were found to be localised on genes coding for *P*₁₋₃ (spot 101), *NP/NA* (spots 100 and 103) and *M* proteins (spot 102). Similar analysis of spots present in the FW virus and not present in the USSR/90 virus confirmed that the detectable oligonucleotide differences between the total RNAs of the two viruses are scattered over the entire genome.

Comparison of the RNA of influenza A/USSR/90/77 (H1N1) virus with RNAs of other H1N1 strains isolated between 1947 and 1956

Subsequent analysis of the oligonucleotide pattern of influenza A/FM/1/47 (H1N1) virus (FM virus, Fig. 1i, j) isolated in 1947 and comparison with the pattern of USSR/90 virus revealed many more differences than were observed between

Table 2 Minimum no. of base changes amongst large oligonucleotides of the RNAs of different H1N1 influenza viruses

RNAs of strains compared	Minimum no. of base changes
A/FW/1/50 and A/USSR/90/77	8
A/FM/1/47 and A/USSR/90/77	34
A/C/1/56 and A/USSR/90/77	28
A/FW/1/50 and A/C/1/56	30
A/I/2/50 and A/USSR/90/77	9
A/FW/1/50 and A/I/2/50	5
A/USSR/90/77 and A/HK/123/77	8
A/USSR/92/77 and A/USSR/90/77	6

To calculate the minimum no. of base changes we compared the oligonucleotide maps of different viral RNAs by coelectrophoresis. Only the large oligonucleotides listed in Table 1 were used for this analysis. In comparing two patterns (I and II) there may be 'missing' spots (present in pattern I and not in pattern II) and 'additional' spots (present in pattern II but not in pattern I). Scoring all missing and additional spots as independent mutational events does not take into account that a single mutation could give rise to both a missing and an additional spot (paired changes). Therefore calculations to obtain an estimate of the minimum number of base changes were made in the following manner. The highest number of missing and additional spots which theoretically could be paired was multiplied by a factor of 1.5 on the statistical assumption that 50% of such paired changes are due to a single and 50% to two separate mutational events; to this number the unpaired changes in either the additional or the missing spots category was added (H. D. Robertson, personal communication). For example, using the oligonucleotide map of FW virus RNA as a reference, comparison with the oligonucleotide pattern of USSR/90 virus RNA revealed 6 missing and 4 additional spots. The minimum no. of base changes was calculated to be $4 \times 1.5 = 6$ (paired changes $\times 1.5$) plus 2 (unpaired changes $\times 1.0$) giving a total of 8. Similar calculations were made for the RNAs of other H1N1 strains using the data summarised in Table 1. The total no. of nucleotides used in the analysis varied between 1,000 and 1,100 for different strains. It should be noted that the values presented here represent rough estimates of the minimum no. of base changes, because oligonucleotides with different sequences may not necessarily be distinguishable in the conditions used. In particular, the variation between viruses with many oligonucleotide differences (for example between A/FM/1/47 and A/USSR/90/77 virus) is likely to be significantly underestimated.

FW and USSR/90 viruses. More than 20 additional USSR/90 virus specific spots (white circles) can be seen in the mixture of oligonucleotides obtained from FM (black circles) and USSR/90 virus RNAs (Fig. 1g, h) (see also Table 1). By comparing 44 large oligonucleotides of FM and USSR/90 viruses it can be calculated that the two sets of oligonucleotides differ by a minimum of 34 nucleotides (for calculation see Table 2). In the absence of nucleotide sequence data, errors of underestimation of differences are much more likely with viruses which are more different genetically. First, it is more likely that oligonucleotides which migrate differently may contain more than one base change. Second, a greater proportion of oligonucleotides, which seem to migrate identically, may possess undetected changes than is the case with closely related strains. Therefore, no attempts were made to express the minimum number of nucleotide changes between the FM

and USSR/90 virus RNAs in per cent of absolute base sequence differences.

Using the same qualitative approach in comparing oligonucleotide maps we determined the relatedness of the RNA of USSR/90 virus and of a late H1N1 (A/C/1/56) virus (C/1 virus) isolated in 1956 (Fig. 2a, b). Again, we calculated a minimum of 28 base changes between the large oligonucleotides of these two viral RNAs (Table 2). A similar number of base changes was observed between the oligonucleotides of FW and C/1 viruses isolated in 1950 and 1956, respectively (Table 2).

Finally, as a control we compared the oligonucleotide pattern of (A/I/2/50) virus (I/2 virus), another 1950 isolate, with the patterns of FW and USSR/90 viruses (Fig. 2c, d). It is evident that the RNA patterns of FW and of I/2 viruses are very closely related (there is a minimum of 5 base changes

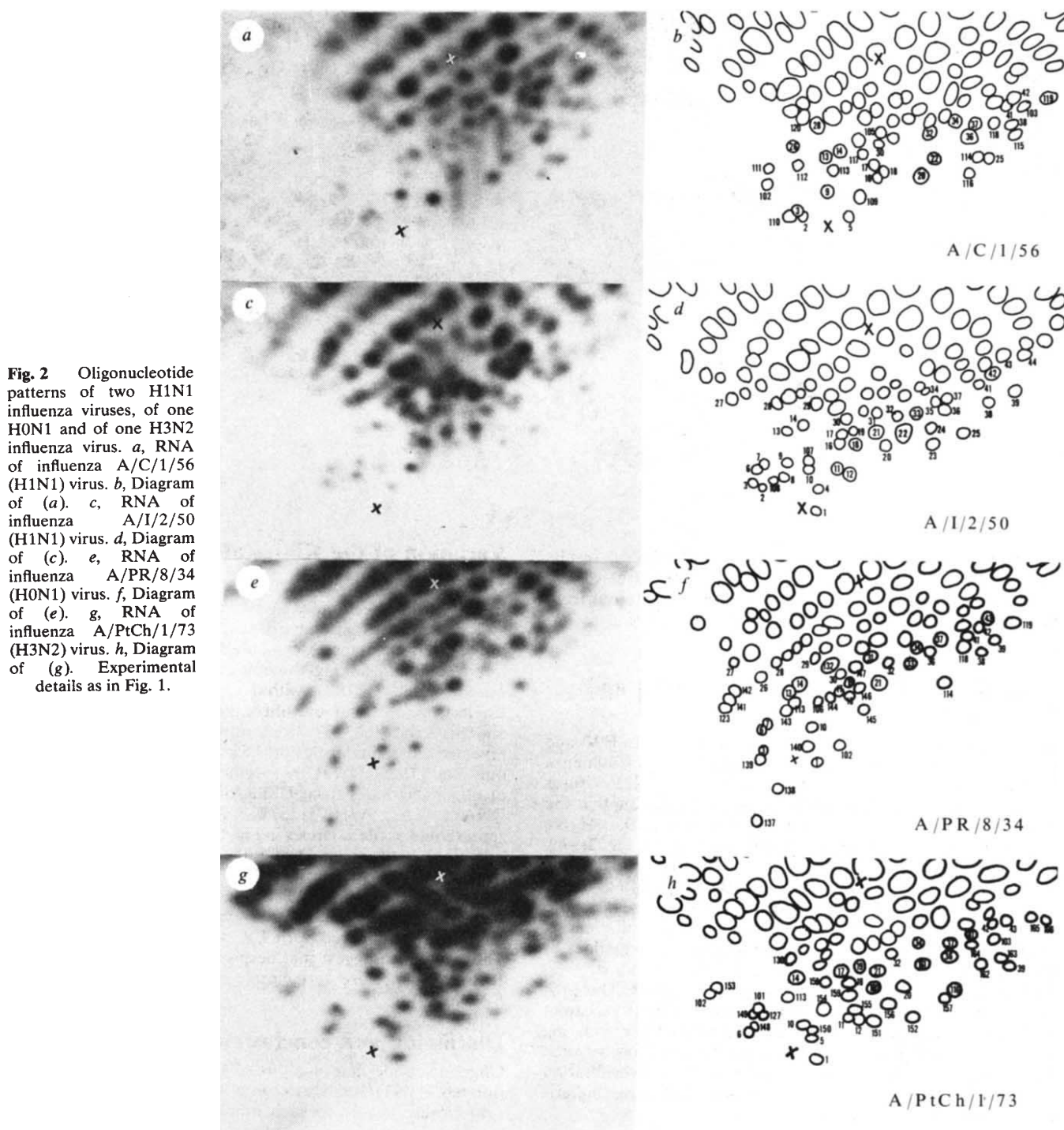
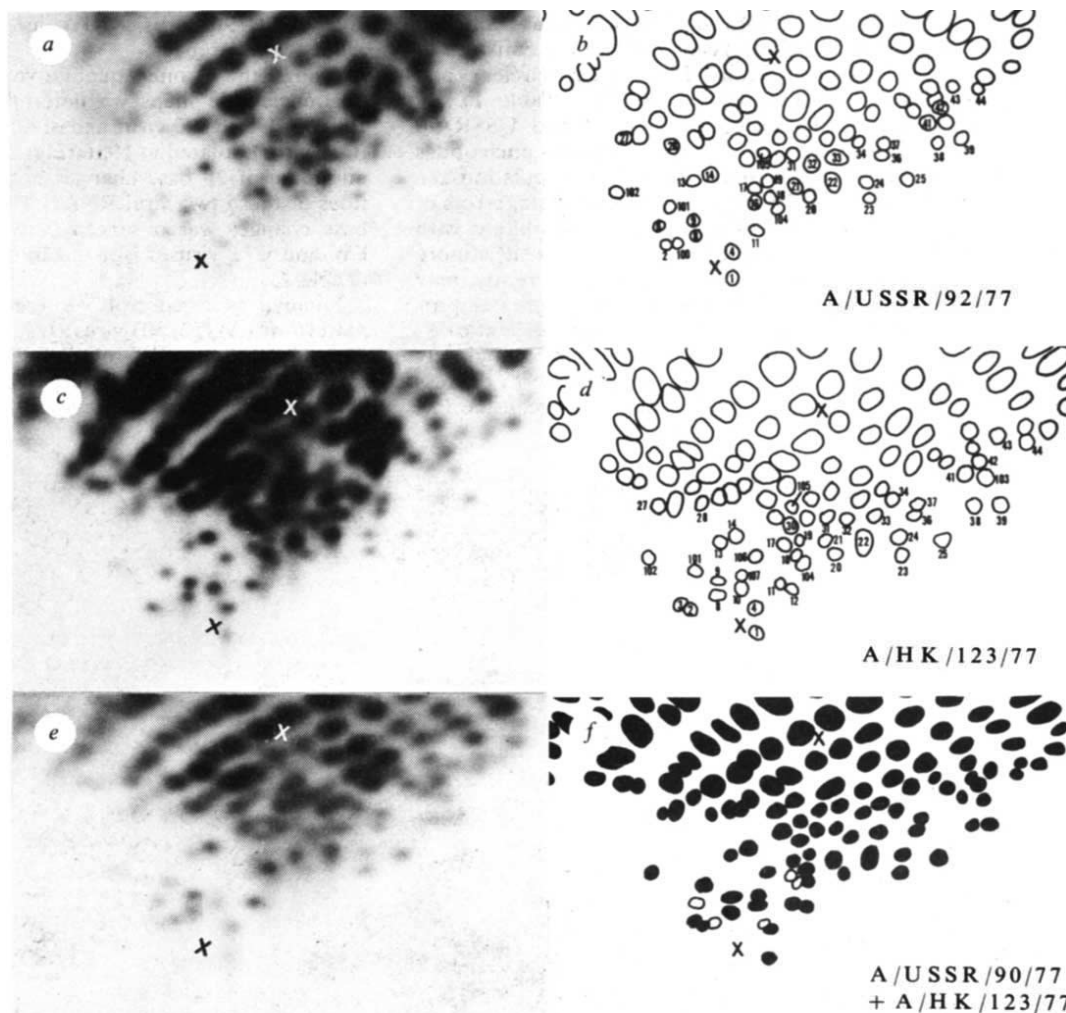


Fig. 3 Comparison of the oligonucleotide patterns of recent H1N1 isolates. *a*, RNA of influenza A/USSR/92/77 virus. *b*, Diagram of (*a*). *c*, RNA influenza A/HK/123/77 virus. *d*, Diagram of (*c*). *e*, Mixture of the RNAs of influenza A/USSR/90/77 (Fig. 1*e*) and A/HK/123/77 viruses. *f*, Diagram of (*e*). Experimental details as in Fig. 1.



between large oligonucleotides) (Table 2). These results confirm our finding that the RNA of USSR/90 virus closely resembles that of strains isolated in 1950 but is different from the RNAs of earlier and later isolates.

Comparison of RNA of H1N1, H0N1 and H3N2 viruses

Comparison of the oligonucleotide maps of the FW and USSR/90 virus RNAs with those of the RNAs of influenza A/PR/8/34 (H0N1) and A/PtCh/1/73 (H3N2) viruses revealed extensive differences in all genes, suggesting that the latter two viruses are not closely related to the 1950 H1N1 strains or to the Russian H1N1 strains (Table 1 and Fig. 2*e-h*). It should be emphasised here that oligonucleotide map analysis is highly valuable for comparing closely related RNA species^{9,11-15} and less valuable for determining the absolute relatedness of RNAs with many base sequence differences. Robertson and Jeppesen⁹ eluted the large oligonucleotides of the three closely related bacteriophage RNAs compared in their study and subjected them to sequence analysis. This procedure allowed direct determination of the extent of variation. Ultimately the same approach would be needed in comparing influenza virus RNAs to evaluate fully the variation between closely and distantly related strains. In addition, hybridisation data may be useful as a preliminary tool for detecting similarities between distantly related RNAs¹⁶.

Variation of the RNAs of three recent H1N1 viruses

In the previous section we showed that two H1N1 viruses (FW and I/2 viruses) isolated in 1950 possess very similar oligonucleotide maps. A comparably small degree of variation was also found among three Russian (H1N1) viruses which were isolated in 1977 within a period of several months. Coelectrophoresis of the oligonucleotides of USSR/90 virus and of A/HK/123/77 virus, another recent H1N1 isolate, revealed close similarities of the patterns. As shown in Fig. 3*f* only 5 additional USSR/90 specific spots appear in the mixture of oligonucleotides from USSR/90 and A/HK/123/77 virus RNAs. (The A/HK/123/77 virus oligonucleotides are represented as black circles in Fig. 3*f*.) Therefore, the degree of homology between the two isolates is comparable with that between USSR/90 virus and the FW strain which was isolated 28 years ago (Table 2). The oligonucleotide map of a third Russian isolate A/USSR/92/77 virus also showed slight variation when compared with FW virus (Table 2, Fig. 3*a, b*). These findings suggest that despite small differences the three Russian isolates are very closely related to each other and to FW virus.

Discussion and conclusion

Oligonucleotide map analysis of the RNAs of three recently isolated H1N1 (Russian) viruses demonstrated that they are very closely related to each other and to two H1N1 viruses

isolated in 1950. In contrast, comparison of the oligonucleotide maps of H1N1 viruses isolated in 1947, 1950 and 1956, revealed extensive genetic differences. These differences in the oligonucleotide maps of the RNAs of viruses isolated within the period of prevalence of one subtype could be due either to the concurrent presence of different variants each of which is genetically conserved, or to multiple mutational events occurring over the entire period. Although the first hypothesis cannot be excluded, for the following reasons we regard the latter explanation to be more likely. First, there is no evidence to show simultaneous prevalence of several markedly different genetic variants belonging to the same subtype. Second, the observation of small differences in the oligonucleotide maps of viruses isolated within a few months of one another suggests that detectable mutations are occurring during epidemic spread over a short period of time.

If we accept the premise that influenza A viruses are subject to repeated mutational events it is extremely difficult to explain why the oligonucleotide maps of strains isolated in 1950 and those of the recent Russian viruses are so strikingly similar. For the reasons stated above it seems unlikely that a 1950 virus survived by normal sequential transmission in the human population without evidence of much more extensive genetic drift. It is also not plausible to speculate that chance back-

mutations accidentally produced a strain so similar to those isolated in 1950.

It seems much more likely that the genetic information in the Russian viruses has been preserved over the last 25–27 years by some unusual mechanism. Although there is no evidence to support the view, it is possible that influenza viruses are capable of latent or persistent infection in man in conditions in which the genetic information of the virus is highly conserved. Alternatively, the genetic information of the virus could have been preserved by sequential passage in an animal reservoir in which influenza viruses replicate without rapid genetic change. (However, in recent unpublished experiments we observed extensive genetic variation among different influenza viruses isolated over a period of a few years from birds and horses.) Finally, it is possible that a 1950 influenza virus was truly frozen in nature or elsewhere and that such a strain was only recently reintroduced into man.

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Where do general anaesthetics act?

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General anaesthetics were found to have no effect on lipid bilayer structures when studied using X-ray and neutron diffraction. Combined gaseous and aqueous phase solubility data suggested that the primary site of action of general anaesthetics has both polar and nonpolar characteristics, and probably involves protein.

THE induction of general anaesthesia, according to most theories^{1–11}, involves a structural change in lipid bilayers of nerve cell membranes. Using both X-ray and neutron diffraction, we have looked for such changes in lipid bilayers. Surprisingly, we can detect no change in bilayer structure at clinical concentrations of general anaesthetics. This has led us to reinterpret the evidence previously used to characterise the anaesthetic site. Many attempts have been made to infer the molecular nature of the site from correlations of anaesthetic potency with solubility in various solvents (for a review, see ref. 12). However, data from experiments in which general anaes-

thetics were administered in the gaseous phase have usually been considered separately from data obtained in aqueous phase experiments. We have combined these data and find an excellent correlation using octanol but very poor correlations using hydrocarbon solvents. From all our results, we conclude that the primary site of action of general anaesthetics has not only apolar but also polar characteristics and probably involves protein.

X-ray and neutron diffraction studies

There is no doubt that sufficiently high concentrations of general anaesthetics will alter membrane structure, and this may account for many of the positive results previously reported with other techniques¹³. Indeed, many general anaesthetics (for example, chloroform and halothane) will dissolve lipid bilayers. It was therefore essential to look for structural changes at clinical concentrations. Also, because we expected only small effects at these low concentrations, it was essential to use the same membrane specimen and diffraction geometry for both control and anaesthetic experiments. Finally, it was necessary to remove the anaesthetic from the sample at the end of the experiment, to check for reversibility. Our solution to these problems was to pass anaesthetics in the gaseous phase over lipid multilayer specimens. In fact, surgical anaesthesia is usually maintained using inhalational anaesthetics, so that the

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corresponding concentrations in the gaseous phase are accurately known. Known partial pressures of three clinically used inhalational anaesthetics (nitrous oxide, halothane and cyclopropane) were humidified using saturated salt solutions and continuously passed through a diffraction chamber¹⁴ containing the lipid multilayers. High resolution diffraction patterns were recorded from these multilayer specimens. We used a mixture of dimyristoyl lecithin and 40% (molar) cholesterol, which is roughly the concentration of cholesterol found in many plasma membranes, including those of nerves^{15,16}. It should be stressed that our bilayers were in a fluid, liquid-crystalline state, as shown by the presence of a single, broad equatorial diffraction band.

Figure 1a shows the result of equilibrating the bilayers with 1 atm of nitrous oxide (which causes general anaesthesia in man¹⁷). It is clear that, within experimental error, there was no change in the bilayer electron density profile. Similarly, 1 atm of cyclopropane (11 times the surgical concentration¹⁷) and 0.093 atm of halothane (12 times the surgical concentration¹⁷) had no effect. Negative results were also obtained for multilayers at different humidities and for multiwalled vesicles in excess water; this was also true for specimens made from egg lecithin alone.

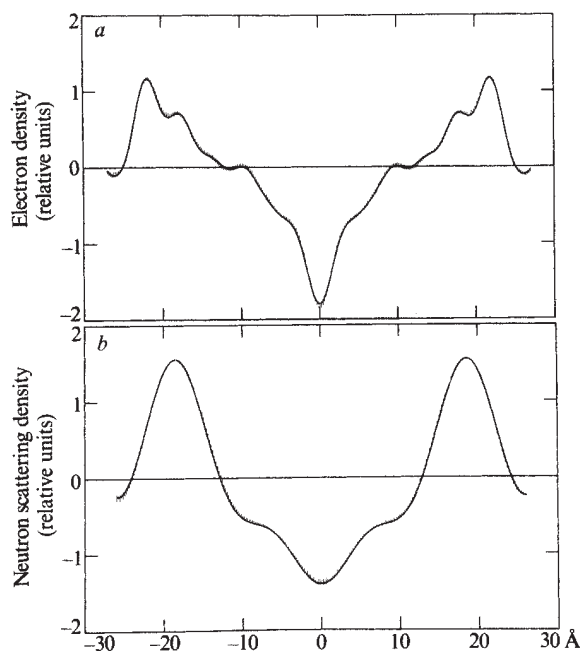


Fig. 1 *a*, Electron density profile of lecithin/cholesterol bilayers in the presence of 1 atm nitrous oxide. *b*, Neutron scattering amplitude density profile of lecithin/cholesterol bilayers in the presence of 1 atm cyclopropane. The vertical bars indicate the 95% mean confidence limits for the control experiments (bilayers at 1 atm helium); the control profiles would lie midway between these limits. Multilayer specimens were made by allowing a solution of the lipids in chloroform/methanol (10:1 v/v) to evaporate slowly on glass substrates. Experiments were carried out at $23 \pm 1^\circ\text{C}$ and 75% relative humidity. The specimens (10–100 μm thick) were pre-equilibrated with the anaesthetic gases for up to 24 h. For the X-ray diffraction experiments, a double-mirror Franks camera was used on an Elliott GX18 rotating anode generator run at 40 kV/50 mA. Otherwise, the experimental procedure was essentially as described previously¹⁹. Structure factors and errors used in the electron density profile (13 orders) were obtained from swelling experiments. The neutron diffraction experiments were carried out essentially as in ref. 20. Errors for the neutron structure factors (four orders) were obtained from $\text{H}_2\text{O}/\text{D}_2\text{O}$ exchange experiments. The profile shown in *b* is for bilayers equilibrated with an $\text{H}_2\text{O}/\text{D}_2\text{O}$ mole ratio of 9:1. A more detailed description of these experiments will be published elsewhere.

If the anaesthetics had caused the choline group of the lecithin molecule (which has an average electron density similar to that of water) to change position¹⁸, this might not have affected the electron density profile^{19,20}. However, the low orders of a neutron diffraction pattern would be particularly sensitive to such a change, but Fig. 1b shows that the neutron scattering density profile is not altered by the presence of 11 times the surgical concentration of cyclopropane.

Relation of anaesthetic potency to solvent/water partition coefficients

As we observed no effects at clinical concentrations on our simple systems, we conclude that structural changes in the lipid bilayer *per se* are unlikely to be responsible for general anaesthesia. This conclusion is strengthened by the findings that clinical concentrations of general anaesthetics do not significantly affect either the fluidity¹³ or mass density²¹ of lipid bilayers. It has been shown, however, that low concentrations of general anaesthetics can lower the melting temperature of cholesterol-free bilayers^{22,23}. That such an effect could be relevant to general anaesthesia seems improbable for at least two reasons. First, if the anaesthetic site is a lipid bilayer poised at a thermal phase transition, then a small increase in temperature should induce anaesthesia^{2,10}. This does not, however, occur with cold-blooded animals. Second, mammalian plasma membranes generally contain high concentrations of cholesterol^{15,16}, and cholesterol suppresses lipid phase transitions²⁴. Thus, a unified mechanism for general anaesthesia is unlikely to include thermal phase transitions in lipid bilayers.

What, then, is the nature of the anaesthetic site? Historically, a general correlation between anaesthetic potency and solubility in olive oil led to the conclusion that the site has a hydrophobic nature^{25,26}. Furthermore, the chemical structure of the anaesthetic seemed to be irrelevant, implying that the site has little specificity (although we now know that chemical specificity may play a part for more complex molecules such as the steroid anaesthetics⁶). On the basis of more recent and precise data, excellent correlations between anaesthetic potency and partitioning behaviour in several solvent systems have been published^{12,27,28}. The correlations using pure, non-polar solvents were nearly as good as the correlation using olive oil²⁸. Furthermore, as the best solvents (benzene, cyclohexane and carbon disulphide) had solubility parameters $\delta = 9 \pm 1$ (cal cm^{-3})^{1/2}, it was proposed that δ was the parameter of choice for characterising the anaesthetic site. Because these recent correlations^{12,27,28} were based on data from mice, due to methodological considerations only inhalational anaesthetics were included. It is striking that many anaesthetic agents (for example, alcohols) which are only conveniently administered in aqueous solution were inevitably excluded from the analysis. However, data for these compounds are available in the literature for newts²⁹ and tadpoles³⁰. To use all the animal data, we converted gaseous concentrations into aqueous concentrations using water/gas partition coefficients.

Quite a different picture emerges when one considers this extended range of data. This is shown by the graphs of Fig. 2. Here we plot the anaesthetic potency (expressed as the reciprocal of that aqueous concentration needed to produce general anaesthesia) against the solvent/water partition coefficient for various solvents. Figure 2a shows the result for oil. Although there is a general correlation, it is clear that at a particular anaesthetic potency, the oil/water partition coefficient for an alcohol is significantly lower than that for other compounds. This trend is shown even more dramatically in Fig. 2b, where the result for hexadecane is plotted. Clearly, the correlation is better for oil, which has some polar characteristics as it is a mixture of glycerides, than for hexadecane, which is a pure hydrocarbon. (A plot using benzene (not shown), a relatively nonpolar solvent, gave a correlation

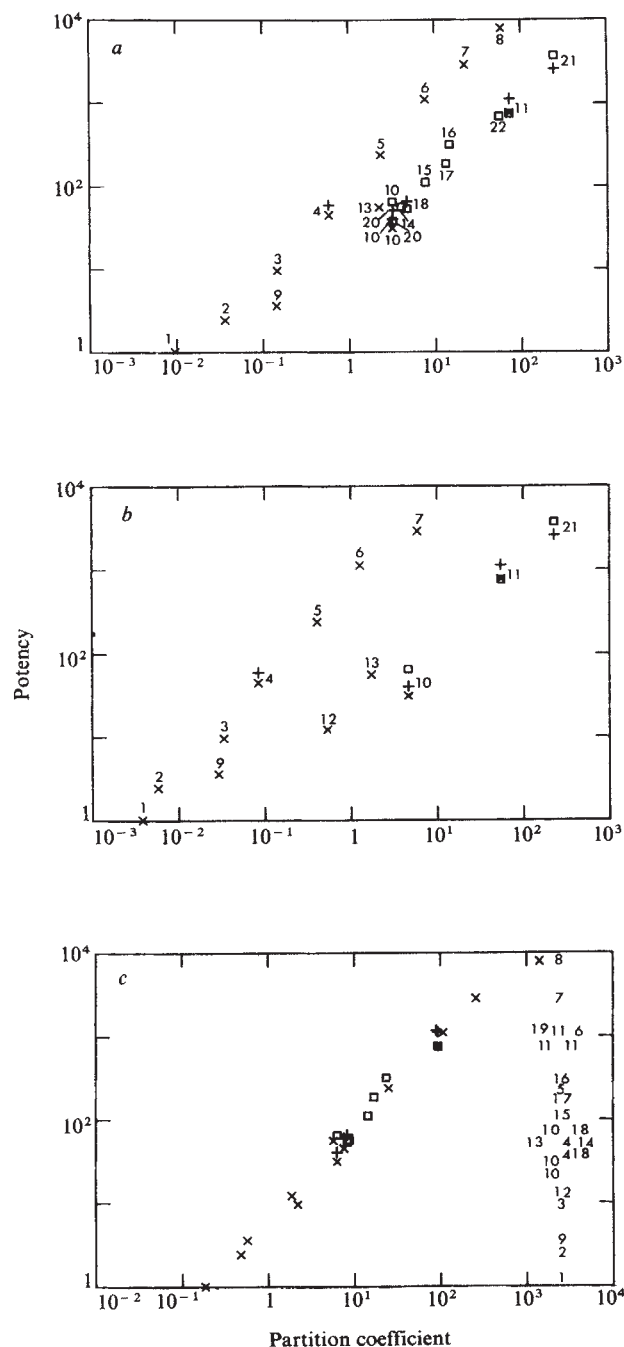
Fig. 2 Anaesthetic potency plotted against solvent/water partition coefficients for three solvents: *a*, vegetable oil; *b*, *n*-hexadecane; *c*, *n*-octanol. The anaesthetic potency is defined as the reciprocal of that concentration (mol l^{-1}) of anaesthetic in the aqueous phase required to produce general anaesthesia. The partition coefficient is defined as the concentration of the anaesthetic in the solvent phase divided by the concentration of the anaesthetic in the aqueous phase at equilibrium. The symbols are: $\square$, mouse; $+$, newt; $\times$, tadpole. General anaesthetic concentrations were obtained from the following sources: mouse, Table 3 of ref. 12; newt, summary of ref. 29; tadpole, Table 5 of ref. 30. When the concentration was given as the partial pressure P (atm) at temperature T (K), we converted it to an aqueous concentration (C_{aq}) using $C_{\text{aq}} = (P\lambda)/(RT)$, where R is the gas constant and λ is the water/gas partition coefficient (Ostwald solubility coefficient)¹². All those anaesthetics for which octanol/water partition coefficients were not available were also excluded from the oil and hexadecane graphs, so that a fair comparison could be made. In addition, we included, where possible, data for the three general anaesthetics used in our diffraction experiments. Partition coefficients were taken from refs 12 and 31–35. In a few cases where solvent/water partition coefficients were not available, these were taken as the ratios of the solvent/gas to water/gas partition coefficients. As so few hexadecane/water partition coefficients are available in the literature, we determined these using ^{14}C labelling (compounds 1, 2, 9), Rayleigh refractometer measurements (compounds 3, 10–13) and gravimetric measurements (compound 21). Anaesthetics are referred to as follows: 1, methanol; 2, ethanol; 3, *n*-propanol; 4, *n*-butanol; 5, *n*-pentanol; 6, *n*-hexanol; 7, *n*-heptanol; 8, *n*-octanol; 9, acetone; 10, ether; 11, chloroform; 12, methyl ethyl ketone; 13, paraldehyde; 14, argon; 15, krypton; 16, xenon; 17, ethylene; 18, nitrogen; 19, sodium pentobarbital; 20, nitrous oxide; 21, halothane; 22, cyclopropane.

slightly better than that for hexadecane but still much worse than that for oil.) This trend suggests that an even more polar solvent might give a better correlation. Figure 2*c* shows that octanol does indeed give an excellent correlation for all compounds and all animals, over an extremely wide range of anaesthetic potency. It is interesting that although both octanol and benzene have solubility parameters $\delta \approx 9$ (cal cm^{-3})^{1/2}, octanol gives an excellent but benzene a poor correlation. Assuming that the remarkable octanol correlation of Fig. 2*c* is not fortuitous, two important conclusions can be drawn: (1) a single site of action is sufficient to account for all these data; (2) this site has solubility characteristics similar to those of octanol.

Characteristics of the anaesthetic site

Taken together, our diffraction and solvent correlation results impose important constraints on future models of anaesthetic action. The X-ray and neutron diffraction data indicate that the lipid bilayer alone is not the anaesthetic site. This suggests that the site is composed, at least in part, of protein. In addition, our solvent correlations show that the site has both polar and apolar characteristics (a conclusion also reached on the basis of thermodynamic arguments³⁶). Both the polar and apolar portions of the site could be on proteins^{13,21}. However, if the primary site of action is in a membrane, then part of the site might be membrane protein and another part the associated lipid^{37,38}. Finally, one might ask why the anaesthetic site is perturbed by concentrations of anaesthetics which have no detectable effect on lipid bilayers. The answer may partly lie in an increased local concentration of anaesthetic molecules at the site, due to the additional attractive forces possible between the anaesthetic molecule and the polar region of the site.

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letters to nature

A galaxy associated with 3C273

THE first QSO for which the redshift was determined, 3C273, has been regarded as the archetypal QSO by astronomers and the public alike. Its importance, however, is not purely symbolic: it is the brightest QSO (and, consequently, one of the best studied); its intrinsic luminosity is nearly an order of magnitude greater than that of any other QSO in its redshift range; it is the only solidly confirmed X-ray source¹ among QSOs; and it is one of the half-dozen or so well-documented superluminal expansion VLBI radio sources². In many ways, 3C273 epitomises the difficulties that led to suggestions that QSO redshifts might not have a cosmological origin. I report here the discovery of a galaxy associated with 3C273.

A recent survey³ of redshifts of a well-defined sample of galaxies near bright, low-redshift QSOs found 8 out of 27 fields for which at least one galaxy had a redshift matching that of the QSO. The probability that a result of this significance or greater could have occurred by chance was shown from the distribution of galaxy redshifts to be less than 1.5×10^{-6} . This is almost incontrovertible evidence in favour of the cosmological nature of QSO redshifts, and also shows that QSOs are more frequently associated with normal galaxies than had generally been assumed. The number of cases for which galaxy-QSO redshift agreements were found is only a lower limit to the number of actual associations within the sample, as the fields over which galaxies were selected were in all cases much smaller than characteristic dimensions of small groups. It is, in fact, possible that most QSOs have companion galaxies.

Although 3C273 was included in this survey, it was one of 10 QSOs for which no galaxies above the magnitude limit of the survey were found within the specified field radius of 45". Bahcall and Bahcall⁴ have indicated a number of galaxies within a radius of about 11' of 3C273; their list, however, was not intended to be comprehensive, as it included only galaxies with an estimated brightness between that of a first-ranked cluster galaxy at the redshift of 3C273 and one 1.5 mag fainter. Furthermore, even within this range, it seems to be incomplete, and, conversely, 'galaxies' number 2 and 3 on their list apparently have perfectly stellar images on the 5-metre telescope photograph published by Greenstein and Schmidt⁵. On that photograph, as well as on a deep red Mauna Kea Observatory plate covering a 3' radius around 3C273, the brightest galaxy evident is some 75" east of 3C273 and is shown on Fig. 1.

Spectroscopic observations of this galaxy, consisting of eight 12-min exposures, were obtained with the photographic image-tube camera mounted on the Cassegrain spectrograph of the Mauna Kea 2.2-metre telescope. The spectrograms were

traced with a 12 μ m square aperture on a PDS micro-densitometer and were reduced in a similar manner to that described in ref. 6, except that ion events and other small defects in individual spectrograms were automatically removed through cross-correlation techniques. The spectrum, after sky subtraction and correction for the system spectral response, is shown in Fig. 2. The 4,000 Å blanketing discontinuity, the H and K lines of Ca II, and the G band are readily apparent at an average redshift $z = 0.1575 \pm 0.0005$, and the spectrum shown in Fig. 2 has been shifted back to the rest frame using this redshift. The redshift is confirmed by the appearance of weak [O II] $\lambda 3,727$ and [O III] $\lambda \lambda 4,959, 5,007$ emission. Within the measurement errors, this redshift is identical to the accepted redshift $z = 0.158$ of 3C273.

The flux in the blue corresponds to an observed B magnitude $M_B = 20.3$. The absolute magnitude, determined for $H_0 = 75 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and $q_0 = 0$ by making the appropriate redshift correction to the flux found within the redshifted B filter profile, is $M_B = -19.9$. Both of these magnitudes are derived from fluxes observed through a 2.75" slit and are, therefore, likely to be overestimated.

Although the presence of [O III] emission in the spectrum of the galaxy is unusual (for example, in the sample of galaxies near QSOs mentioned above³, only one out of 17 galaxies with

Fig. 1 Identification chart showing the region around 3C273, which is centred. The galaxy discussed here is marked at the left. North is at the top, east to the left, and the figure is 4' wide.

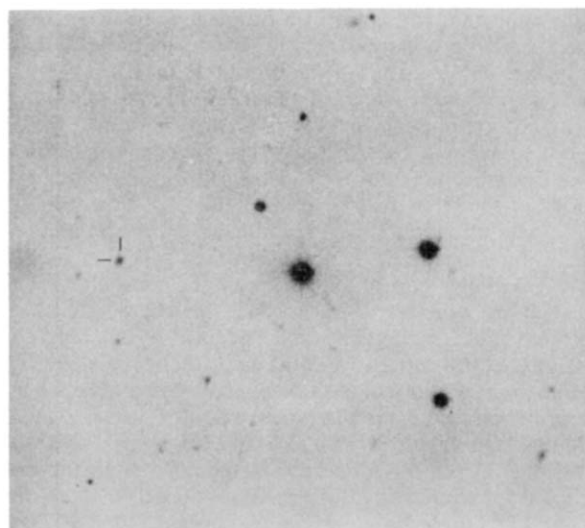
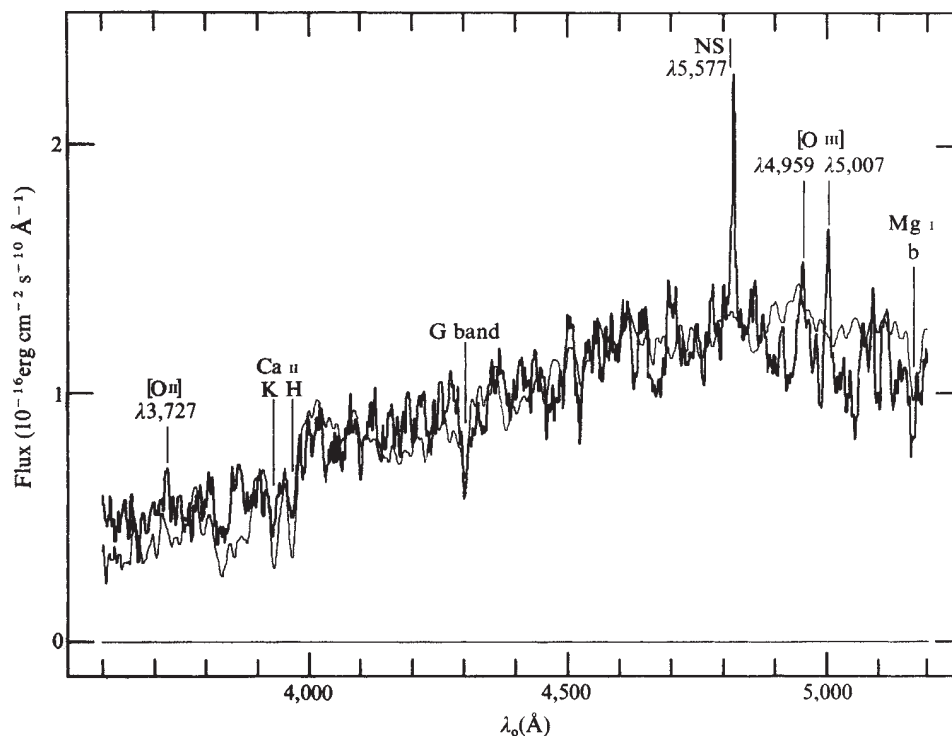


Fig. 2 The spectrum of the galaxy 75" east of 3C273, reduced to the rest wavelength system. Fluxes have been corrected to a local frame in which 3C273 would have zero redshift. The lighter trace is the spectrum of the nucleus of M31, shown for comparison. The bluer continuum energy distribution, the reduced break at 4,000 Å, and the presence of weak emission lines all point to an earlier integrated spectral type for the galaxy than for the centre of M31.



the necessary wavelength coverage had detectable [O III] λ 5,007 emission), there is no obvious way that the proximity of the galaxy to 3C273 can be blamed. At a minimum distance from the galaxy of about 200 kpc, 3C273 fails by some three orders of magnitude to produce sufficient Lyman continuum flux to ionise the gas in the galaxy for densities $N_H = 1$ or greater.

Finding a galaxy associated with 3C273, which has a flat radio spectral index, helps temper somewhat a curious asymmetry in the results of the survey of galaxies near QSOs³. Although QSOs known to have steep radio spectral indices only slightly outnumbered those with flat spectral indices (10 steep, 7 flat) in the original sample, five of the eight QSOs for which associated galaxies were found had steep spectral indices, while only one had a flat index. The radio spectral index is known to correlate with other observed properties, notably the nature of the magnitude-redshift relationship^{7,8}, the steepness of the cosmological density evolution^{9,10}, and apparently the tightness of the correlation between continuum luminosity and emission-line equivalent width¹¹. Although flat-spectrum QSOs still seem under-represented among those that have associated galaxies, it would be premature to claim that there is strong evidence for such a correlation.

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Hard X-ray observations of cosmic γ -ray sources

THE publication¹, by workers using the COS B satellite, of a list of 13 discrete cosmic γ -ray sources has aroused considerable interest. Attention has mainly been focussed on attempts to identify all or some of these sources with known X-ray sources^{2,4}, optical sources^{5,6}, radio sources⁷ and H II regions⁸. In most cases the large COS B error boxes have made this a difficult task. We report here on hard X-ray studies of these γ -ray error boxes and suggest that unresolved sources of this type could account for the galactic disk γ -ray emission.

Our survey was carried out using data accumulated over the last 3 yr by the Imperial College scintillation telescope (ST) on Ariel 5. The instrument covers the spectral range 26-1,200 keV and has a field of view of FWHM 8°. Its detailed construction and operation are described elsewhere⁹. For this study we concentrated on two spectral bands: 260-570 and 570-1,230 keV.

A summary of our results is presented in Table 1. It can be seen that, with the exception of the Crab Nebula (CG185-5), the only source showing an indication of a hard X-ray flux is CG135+1. The total significance of this flux is 3.2 SD. Of the remaining sources we can take CG312-1 as a typical example. This γ -ray source has been tentatively identified with 4U1416-62 (ref. 3), and we can plot an overall spectrum using this identification, our upper limits and the COS B flux value. This is shown in Fig. 1a. The simplest spectrum fitting all these data is a power law of index -2.0 which is similar to that seen to fit data for the Crab Nebula over the same spectral range (see Fig. 1b).

The Uhuru identification combined with our upper limits clearly excludes any flatter type of spectrum over the energy range 2 keV-1 MeV. This argument applies to all the sources except CG135+1 which we discuss below.

If we assume that each of the COS B sources is associated with a known X-ray source, then considering all the catalogued galactic X-ray sources covered by the COS B survey, we find that ~13% of these catalogued sources should have similar

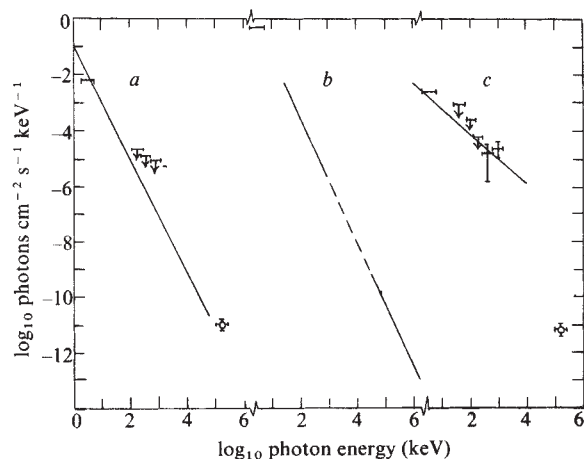


Fig. 1 *a*, COS B and ST data for CG312-1 with a connecting power law of $0.1 E^{-2.0}$. The Uhuru flux value¹¹ of the suggested X-ray counterpart³ is also shown. *b*, Power law fits with index -2.1 to the COS B and ST data for Crab Nebula from refs 13 and 10. The Uhuru flux¹¹ is also shown. *c*, COS B and ST data for CG135+1 with a power law fit of index -0.9 in the range 2-1200 keV. The Uhuru flux¹¹ for the suggested X-ray identification 4U0241+61 is also shown.

power law spectra extending to γ -ray energies. Integrating over a $\log N - \log S$ distribution such as that published in the 4th Uhuru catalogue¹¹ it is possible to estimate the contribution of these sources to the galactic γ -ray disk emission above 100 MeV. This gives 5×10^{-5} photons $\text{cm}^{-2} \text{s}^{-1} \text{radian}^{-1}$, which is very close to the average measured COS B flux¹² of 4×10^{-5} photons $\text{cm}^{-2} \text{s}^{-1} \text{radian}^{-1}$. So the possibility that this disk emission is built up of unresolved sources of this type seems quite feasible. One possible mechanism which could provide emission over the X-ray and γ -ray region is the model of Fabian *et al.*¹⁴ for Cyg X-3. In this model the hard X rays come from inverse Compton scattering of the stellar photons and the γ -ray flux from inverse Compton scattering of the soft X rays. The necessary source of X rays and relativistic (~ 100 MeV) electrons could be produced as suggested in the young pulsar model of Lamb *et al.*¹⁵.

That this idea of a single power law spectrum covering the whole range from 1 keV to 100 MeV may well be too simple, is shown by our measurements on CG135+1 (Fig. 1c). If the

identification with 4U0241+61 is correct, then at least one break in the spectrum is essential to fit all the data. Interestingly, there is great similarity between this unusually flat X-ray spectrum and the high latitude source 2A1348+700 (ref. 16). This source has been identified with a Seyfert galaxy MKN279, although some doubt has been expressed over this identification (J. Burnell, personal communication).

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Origin of MeV-excess in the energy spectrum of diffuse cosmic γ rays

THE origin of the diffuse cosmic γ radiation has been widely discussed. It is either interpreted as the result of interactions in intergalactic space or as the result of the superposition of many unresolved external galaxies. Strong support for the latter hypothesis is given by our measurements of the energy spectrum of the Seyfert galaxy NGC4151 up to 10 MeV with the MPI Compton telescope¹ which are reported here.

Diffuse cosmic X- and γ -radiation has been observed during the past decade after much experimental effort by many groups. The existence of this radiation in the X-ray range has been established for several years. Below 100 keV, the radiation is isotropic to within 4% in angular intervals of 10° (refs 2, 3) which is strong evidence for an extragalactic origin. The continuation of the diffuse cosmic X-ray spectrum towards γ -ray energies does not follow a simple power law. There seems to be a bump in the spectrum at energies of a few MeV. All existing measurements in this range tend to converge and support the existence of this bump. At higher γ -ray energies the spectrum is very steep. Figure 1 shows a compilation of the most recent γ -ray measurements (from refs 4, 5).

Various models have been proposed to explain the bump (for reviews see refs 6-8). Much interest has been devoted to two cosmological models^{9,10} in which most of the γ radiation above 1 MeV comes from the decay of π^0 -mesons produced in cosmological epochs, either by interactions of cosmic rays with intergalactic gas or by matter-antimatter annihilation in a baryon-symmetric universe. In these models, γ rays were produced at epochs when the matter density of the Universe was much higher than at present. The resulting γ -ray spectrum is, in the present epoch, redshifted substantially by the expansion of the Universe so that the characteristic π^0 -peak at 67.5 MeV is displaced to a few MeV.

Table 1 Summary of Imperial College hard X-ray observations of COS B γ -ray sources

γ -ray source	Hard X-ray flux ($\times 10^{-5}$ photons $\text{cm}^{-2} \text{s}^{-1} \text{keV}^{-1}$)		Date of observation
	260-570 keV	570-1,230 keV	
CG65+0	≤ 4.3	≤ 3.1	June 1975
CG75+0	≤ 3.4	≤ 2.4	Nov. 1976
CG78+1	≤ 1.9	≤ 1.3	Nov. 1976
CG121+3	≤ 3.2	≤ 2.3	July 1975
CG135+1	2.1 ± 1.8	2.5 ± 1.3	July 1975
CG176-7	≤ 2.9	≤ 2.0	Sept. 1975
CG185-5	3.0 ± 0.8	1.4 ± 0.6	April 1975
CG189+1	≤ 2.8	≤ 2.0	Sept. 1975
CG195+4	≤ 2.5	≤ 1.8	Sept. 1975
CG263-2	≤ 2.5	≤ 1.7	Feb. 1978
CG312-1	≤ 1.5	≤ 1.0	Nov. 1977
CG327-0	≤ 4.4	≤ 3.1	Feb. 1976
CG335+0	≤ 2.9	≤ 2.0	Sept. 1976

All upper limits are $2 \times \text{s.d.}$

The observation of the Seyfert galaxy NGC4151 at γ -ray energies¹ strongly supports the hypothesis that the diffuse cosmic γ radiation results from the superimposition of many unresolved galaxies of the same type as NGC4151. (A similar treatment was carried out in refs 11–13 before actual intensities from Seyfert galaxies were available at γ -ray energies.)

Figure 2 shows the energy spectrum of NGC4151 plotted between 2 and 10 MeV. The measurements with the Compton telescope are in good agreement with a power law extrapolation from X-ray energies up to about 3 MeV. Above this energy the spectrum is considerably steeper. Strictly speaking, the measurements of the Compton telescope are only an upper limit because all excess counts of the telescope observed during the transit of NGC4151 through its aperture of $\approx 40^\circ$ FWHM were attributed to this one source. The consistency of the γ -ray data with the X-ray data, however, may indicate that this procedure was correct. (The discrepancy between the MISO γ -ray measurements¹⁴ and the MPI measurements may be due to the fact that the former measurements were evaluated without using any spectral information of the data.)

The measured spectrum of NGC4151 has been fitted by a power law $4.5 \times 10^{-3} E^{-1}$ photons $\text{cm}^{-2} \text{s}^{-1} \text{keV}^{-1}$ below

Fig. 1 The energy spectrum of the diffuse cosmic γ -ray spectrum (from refs 4, 5). The power law fits to the observed flux values below and above 3 MeV have the same spectral index as the observed energy spectrum of NGC4151. (The dashed spectrum of Fichtel *et al.*⁵, is nearly identical with the power law fit between 35 and 200 MeV.)

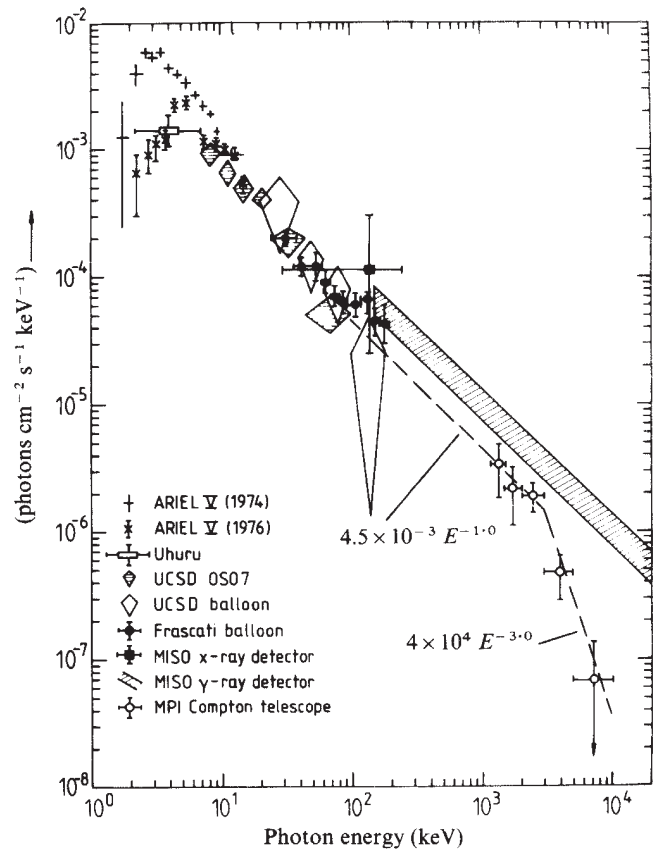
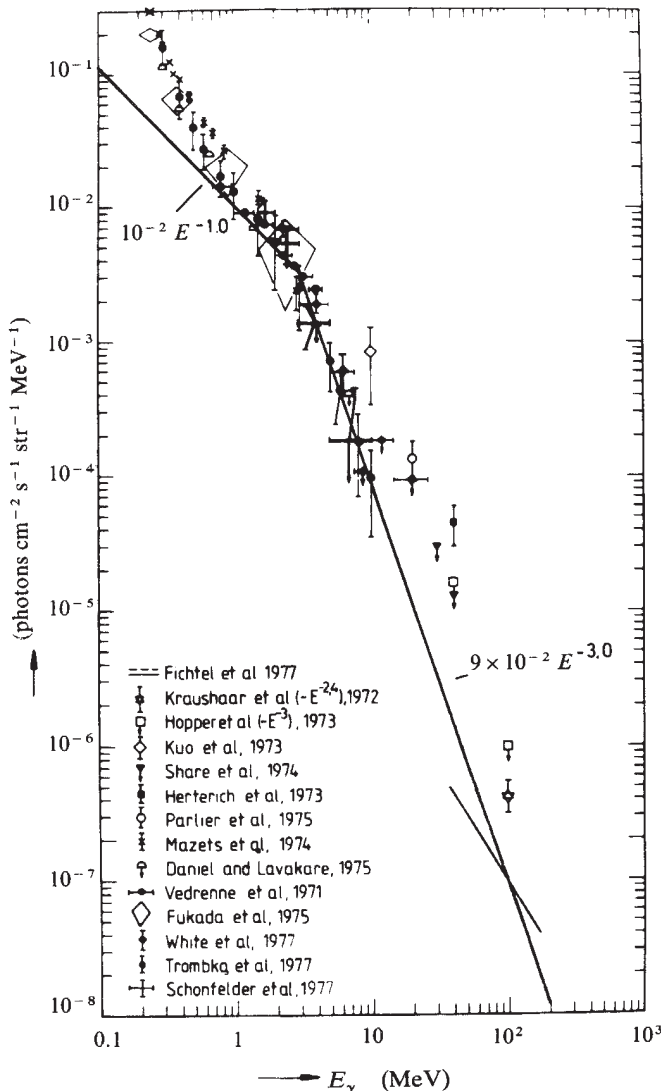


Fig. 2 The energy spectrum from the direction of NGC4151 at γ -ray energies as determined by the MPI Compton telescope¹ is compared with X-ray data of the same source (adapted from ref. 15).

3 MeV and $4 \times 10^{-4} E^{-3}$ photons $\text{cm}^{-2} \text{s}^{-1} \text{keV}^{-1}$ above 3 MeV (dotted lines in Fig. 2). Spectra with the same exponents have also been drawn into the diffuse cosmic γ -ray spectrum (Fig. 1), illustrating that its spectral shape above 1 MeV can be nicely fitted by these two power laws.

As far as the spectral shape is concerned, galaxies of the same type as NGC4151 could, therefore, explain at least the bump at MeV energies if not the total diffuse cosmic γ -ray spectrum above 1 MeV (of course, the true NGC4151 spectrum could be steeper than $\sim E^{-3}$ above 3 MeV). A spectral shape flatter than $\sim E^{-3}$ would cause difficulties. The spectral index above 3 MeV will be obtained soon when the COS B observation of this region has been evaluated. Only if the measured flux at 100 MeV from NGC4151 is stronger than that from the Crab will its spectrum be flatter than $\sim E^{-2.8}$.

To estimate the required density of galaxies, the diffuse cosmic γ -ray flux $I(E)$ ($\text{MeV cm}^{-2} \text{s}^{-1} \text{sr}^{-1} \text{MeV}^{-1}$) is expressed as

$$I(E) = \frac{Q(E)nR_{\max}}{4\pi} \quad (1)$$

$Q(E)$ ($\text{MeV s}^{-1} \text{MeV}^{-1}$) is the luminosity at energy E , $n(\text{Mpc}^{-3})$ is the required density of galaxies. When $R_{\max} = 1/2(c/H_0)$ equation (1) holds to within a factor of 2 for the popular models of Friedman cosmology if no significant evolutionary effects are assumed ($H_0 = 50 \text{ km s}^{-1} \text{Mpc}^{-1}$).

From the fit to the observed flux values of Fig. 1 an emissivity $B(E) = Q(E)n = 4\pi I(E)R_{\max}^{-1}$ is derived, which is

$$B(E) = 1.4 \times 10^{-29} E^{-1} \text{ photons cm}^{-3} \text{s}^{-1} \text{MeV}^{-1}$$

below 3 MeV. Its integrated value is

$$B_{(<3\text{ MeV})} = 1.8 \times 10^{39} \text{ erg s}^{-1} \text{ Mpc}^{-3}$$

The luminosity of NGC4151 below 3 MeV is

$$Q_{(<3\text{ MeV})} = 10^{45} \text{ erg s}^{-1}$$

for a distance of 20 Mpc. The required density of galaxies is, therefore, $n = 1.8 \times 10^{-6} \text{ Mpc}^{-3}$. According to Elvis *et al.*¹⁶ the space density of X-ray Seyferts with luminosities (2–10 keV) between $Q_{X\min}$ and $Q_{X\max}$ can be expressed as

$$n_X[\text{Mpc}^{-3}] = \int_{Q_{X\min}}^{Q_{X\max}} 2.0 \times 10^{37} Q_X^{-2} dQ_X \quad (2)$$

with Q_X measured in erg s^{-1} . The density of X-ray Seyferts with (2–10 keV)-luminosities above $10^{42.85} \text{ erg s}^{-1}$, the value of NGC4151, is $2.8 \times 10^{-6} \text{ Mpc}^{-3}$. X-ray Seyferts, therefore, could explain the observed diffuse cosmic γ -ray flux, if nearly all of them have similar power and similar spectra as NGC4151 at γ -ray energies. If there is proportionality between γ -ray power and X-ray power in these objects, only 13% of all X-ray Seyferts are needed. For the very early Seyferts the break in the spectrum at 3 MeV will be redshifted. If, therefore, redshift and/or evolution are taken into account the picture will change slightly; however, the conclusion of this paper will not change qualitatively. But the spectrum of only one single external galaxy has been observed at γ ray energies and more observations are strongly needed.

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On the identification of celestial γ -ray sources

THE observations from COS B have provided a new and more detailed picture of the high energy γ -ray emission from the Galaxy. We discuss here the first catalogue with 13 localised sources which has been compiled^{1,2} and how the list should lengthen in the near future, as the data analysis progresses.

The first positive γ -ray observation was made by Kraushaar *et al.*³ with the OSO 3 satellite. They were able to identify a line-like emission from the galactic disk, while the poor angular resolution of the instrumentation practically ruled out the

possibility of detecting point-like sources. The most economical interpretation of the data was that of a diffuse emission by π^0 -decay following the interactions of cosmic rays with the interstellar matter³. The SAS 2 data⁴ have indicated a more structured spatial distribution of the γ -ray emission and the presence of few discrete sources. The general interpretation, however, was unchanged and correlations between the emission features and the distributions of cosmic rays and interstellar gas were suggested^{4,5}. On the other hand, the point sources were still considered to have a secondary role in this picture.

Publication of the first COS B catalogue^{1,2} has triggered a different way of thinking. The role of the sources is now more emphasised⁶ and several attempts to identify some with galactic or extragalactic objects are being proposed. However, a critical point of view is necessary to obtain productive results and to avoid spurious correlations. The positional error given in the first COS B catalogue¹ is meant to express only approximately the uncertainty existing in positioning the centre of the observed excess of γ rays and it does not define an 'error box' with the current meaning of a quantitative boundary in terms of standard deviations.

Amongst the galactic objects, supernova remnants (SNRs), H II regions and pulsars are favourite candidates. Lamb⁷ has discussed a relationship between SNRs and γ sources. Because of the large indetermination associated with the position of the γ sources, a correlation based on a pure positional occurrence loses its significance and therefore Lamb puts a constraint on the general sample of SNRs calling for small diameters (less than 15 pc), as indication of the 'youthfulness' of the remnants. Using the Ilovaisky and Lequeux⁸ catalogue, Lamb concluded that a non-chance correlation can be found on the basis of three coincidences (CG78+1 with G78.1+1.8; CG327-0 with G326.2-1.7 or G328.4+0.2; CG333+0 with G333.0+0.8).

However, on the basis of observations more recent and detailed than those quoted by Lamb, the radio source G333.0+0.8 has been rejected as a SNR and has been recognised as a H II region because of its thermal spectrum and the presence of the H109 α line⁹. Clark and Caswell¹⁰ have compiled a new list of SNRs and give new estimates of distances and diameters. The remaining SNRs discussed by Lamb have diameters greater than the youthfulness limit of 15 pc; in particular, the diameter values quoted for G78.1+1.8 and G326.2-1.7 are above 20 pc. It seems, therefore, that this 'class to class' correlation is strongly weakened. This accident seems to favour the identification of γ -sources with H II regions, adding to the two suggested by Strong¹¹ (CG135+1 with W3, CG189+1 with NGC2175) the new candidate G333.0+0.8. However, the difficulty associated with having a cosmic ray density four orders of magnitude higher than that generally accepted in the solar neighbourhood remains unsolved, even if it could be bypassed by postulating a strong source of high energy particles inside or near the H II region. We also note that a variable radio source¹² and a QSO¹³ have been discovered near the position of CG135+1.

The two examples discussed above show the results that could be expected when two disk populations are compared on a purely positional basis, and when one of the two has positional indeterminations of the order of those which can be associated with the CG sources.

More characteristic features have to be considered such as spectral properties (model dependent) and/or temporal behaviour of the emission (observational). In fact, time analysis has been the decisive factor in determining the only two firmly identified sources, CG185-5 and CG263-2, whose counterparts are the pulsars PSR0531+21 (Crab) and PSR0833-45 (Vela), respectively. In this respect, pulsars seem to be a privileged class, with radio observations providing the input for the possible identification at γ ray energies. We stress, however, the necessity of having accurate measurements of the radio-pulsar parameters and an adequate approach in handling the weak γ -ray signals¹⁴.

In conclusion, we stress that identifications based on positional 'class comparison' are generally premature and inconclusive, causing the errors associated with the position of γ sources. A more useful approach is to identify individual sources by looking for definite time-structures appearing at different wavelengths and/or peculiar energy spectra.

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Quark stars with 'realistic' equations of state

THE possibility of a phase transition between nuclear matter and quark matter has been discussed in recent work¹⁻¹³. Free quarks would presumably appear above a critical density ρ_q , which is greater than the typical density $\rho_n \approx 2.5 \times 10^{14} \text{ g cm}^{-3}$ within ordinary atomic nuclei. Such a transition might allow stable collapsed stars to exist wherein much of the matter is in the form of quark matter (quark stars). The maximum masses of collapsed stars have been investigated for the extreme case of $\rho_q \approx \rho_n$ and hard equations of state for quark matter⁵. However, it has been argued^{6,8,9} that some equations of state for quark matter require $\rho_q \gg \rho_n$ and are therefore unlikely to permit the existence of stable quark stars. We investigate here the properties of collapsed stars in the context of another class of quark-matter models that has recently been proposed¹¹⁻¹³. These models¹², which are consistent with all experimental nuclear and high-energy physics data, are based on a quantum

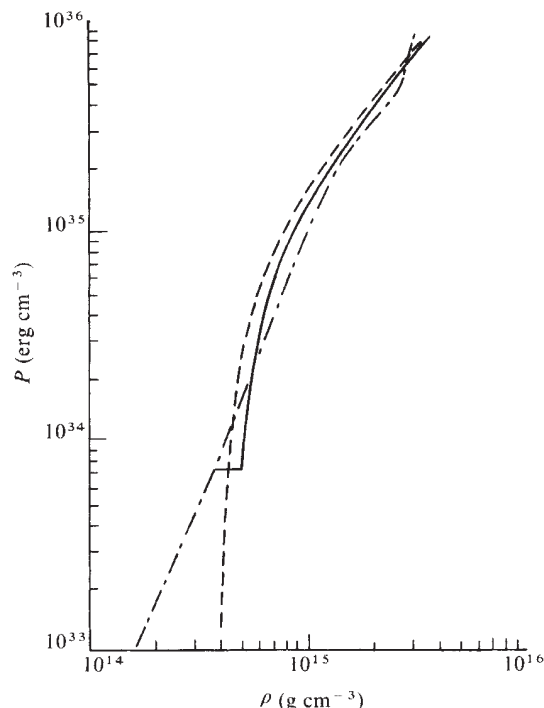


Fig. 1 Equation of state (pressure P plotted against density ρ) for the BJVH nuclear-matter potential¹⁵ (---). Quark-matter equation of state for $B = 56 \text{ MeV fm}^{-3}$ and $\mu = 85 \text{ MeV}$ (ref. 12), joined to the BJVH equation of state by a first-order phase transition (—). Quark-matter equation of state for $B = 0 \text{ MeV fm}^{-3}$ and $\mu = 276 \text{ MeV}$ (— · —). This value of μ was chosen to yield a second-order phase transition to the BJVH equation of state at $\rho_q = 4.5 \times 10^{14} \text{ g cm}^{-3}$.

chromodynamic treatment of quarks that are assumed to be of low mass ($\leq 100 \text{ MeV}$ for the lightest quarks). We find that stable quark stars are possible (compare with refs 6, 8, 9). However, we also conclude (compare with refs 5, 14) that such stars need not have significantly different macroscopic properties from those of neutron-star models¹⁵⁻¹⁷ based on conventional nuclear-matter equations of state.

The quark-matter models that we use contain two parameters¹²: μ , which describes the strength of the quark-quark interaction; and a 'bag constant' B , which is essentially

Table 1 Properties of the stellar models

B (MeV fm^{-3})	μ (MeV)	Nuclear potential	Phase transition type	ρ_q (10^{14} g cm^{-3})	ρ_o (10^{14} g cm^{-3})	$\rho_{c,\text{max}}$ (10^{14} g cm^{-3})	M_{max} ($M_\odot$)	I_{max} (10^{44} g cm^2)	z_{max}
0	273	R	I	2.9	4.0	20	1.92	22	0.40
0	275	R	II	4.4	4.4	20	1.89	21	0.39
0	273	BJVH	I	3.0	4.0	20	1.92	22	0.40
0	276	BJVH	II	4.5	4.5	20	1.89	21	0.40
0	295	TI3	I	3.2	6.0	25	1.73	18	0.34
0	315	TI3	I	4.8	8.7	{4.7} {20}	{1.83} {1.71}	{36} {23}	{0.22} {0.28}
56	85	R	I	3.8	5.0	25	1.69	15	0.39
56	95	R	II	5.4	5.4	25	1.69	15	0.38
56	85	BJVH	I	3.1	4.9	25	1.69	15	0.38
56	102	BJVH	II	5.8	5.8	25	1.68	15	0.38
56	170	TI3	I	2.8	5.9	25	1.61	14	0.33
56	230	TI3	I	4.2	8.2	{8.2} {25}	{2.24} {1.60}	{44} {19}	{0.30} {0.28}

ρ_q and ρ_o are the minimum and maximum densities across the phase transition, respectively; $\rho_{c,\text{max}}$ is the maximum central density for a stable star and, within the context of each model, is calculated to an accuracy of $\pm 3 \times 10^{14} \text{ g cm}^{-3}$; M_{max} is the maximum mass of a stable star ($\pm 0.01 M_\odot$); I_{max} is the maximum moment of inertia of a stable star ($\pm 1 \times 10^{44} \text{ g cm}^2$); and z_{max} is the maximum surface redshift of a stable star (± 0.01). In two cases there are two sets of maxima, the first corresponding to neutron stars and the second to quark stars (see Fig. 2); $\rho_{c,\text{max}}$ for the neutron stars is calculated to an accuracy of $\pm 0.1 \times 10^{14} \text{ g cm}^{-3}$.

an undetermined positive constant in the thermodynamic potential. For suitably chosen values of μ or B , one may obtain a wide range of values of ρ_q or eliminate the existence of a phase transition altogether. Models with $\rho_q \leq \rho_n$ are excluded by the lack of observable phase-transition effects in ordinary atomic nuclei¹². Models with $\rho_q \gg \rho_n$ (or with no phase transition at all) cannot be excluded, but the consequences for the physics of collapsed stars would then be unimportant⁹. We shall, therefore, restrict our attention to models where $\rho_q \approx (1.1-2.5)\rho_n$.

For simplicity, we concentrate on two cases: $B = 56 \text{ MeV fm}^{-3}$, which is about the value suggested by the 'bag model' of the hadrons¹⁸, and $B = 0 \text{ MeV fm}^{-3}$. At lower densities ($\rho \leq \rho_n$) we use three different nuclear-matter equations of state (and corresponding internal energies): (1) an equation of state¹⁹ based on the Reid potential²⁰, which is relatively soft ($dP/d\rho$ is relatively small); (2) the equation of state given by Malone *et al.*¹⁵ for their VH potential, which is of intermediate stiffness; and (3) the equation of state given by Pandharipande and Smith²¹ for their tensor-interaction potential 3, which is relatively hard. These three equations of state are denoted here as R, BJVH, and TI3, respectively.

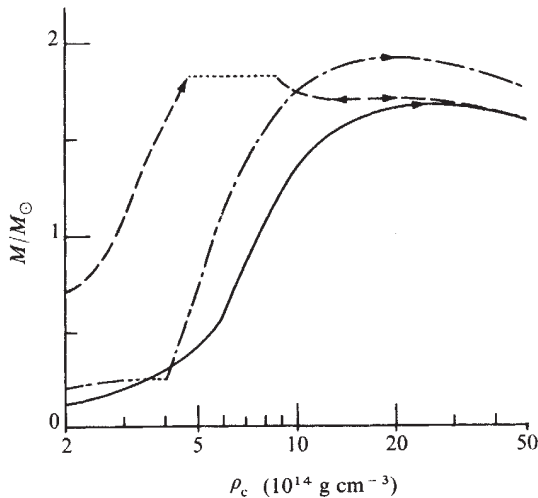


Fig. 2 Mass M plotted against central density ρ_c , for three illustrative cases of stellar models based on quark-matter equations of state joined to nuclear-matter equations of state (see text). The dotted lines denote forbidden intervals of ρ_c corresponding to the density intervals across first-order phase transitions. The arrows point away from regions of dynamic stability and toward regions of instability. $B = 0 \text{ MeV fm}^{-3}$, $\mu = 315 \text{ MeV}$ joined to the TI3 equation of state²¹ by a first-order phase transition (---). Note the discrete region of stable quark stars for $1.3 \times 10^{15} \leq \rho_c \leq 2.0 \times 10^{15} \text{ g cm}^{-3}$. $B = 0 \text{ MeV fm}^{-3}$, $\mu = 273 \text{ MeV}$ joined to the R equation of state¹⁹ by a first-order phase transition (-.-.-). $B = 56 \text{ MeV fm}^{-3}$, $\mu = 102 \text{ MeV}$ joined to the BJVH equation of state¹⁵ by a second-order phase transition (—).

For a given value of B and a given equation of state and internal energy for nuclear matter, the equation of state and internal energy is specified at all densities by choosing a value for μ . The equations of state of nuclear and quark matter must be joined by a standard thermodynamic construction, which requires knowledge of the internal energy as a function of density for each state of matter^{6,8}. For large values of μ there will be no phase transition at any reasonable density, and for small values there will be a first-order phase transition across a range of densities $\rho_q \leq \rho \leq \rho_n$. There may be a second-order phase transition at a unique value of μ , in which case the value of ρ_q will be larger than for a first-order transition.

We have calculated equations of state for both values of B , for all three chosen nuclear-matter equations of state, and for both first-order and second-order phase transitions between nuclear and quark matter. The results of some of these calculations are shown in Fig. 1. In each case, we have then used

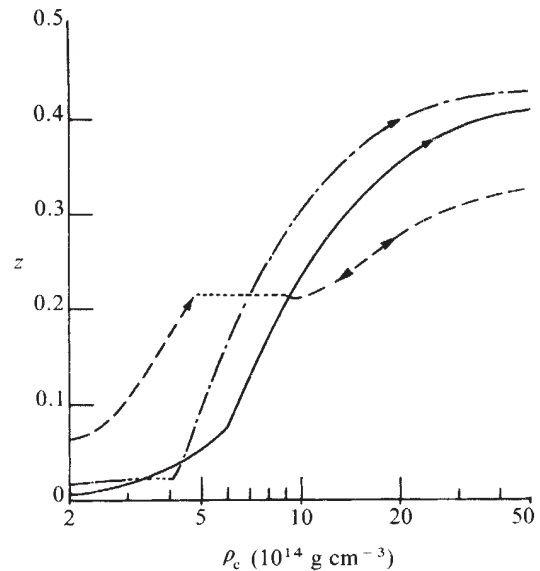
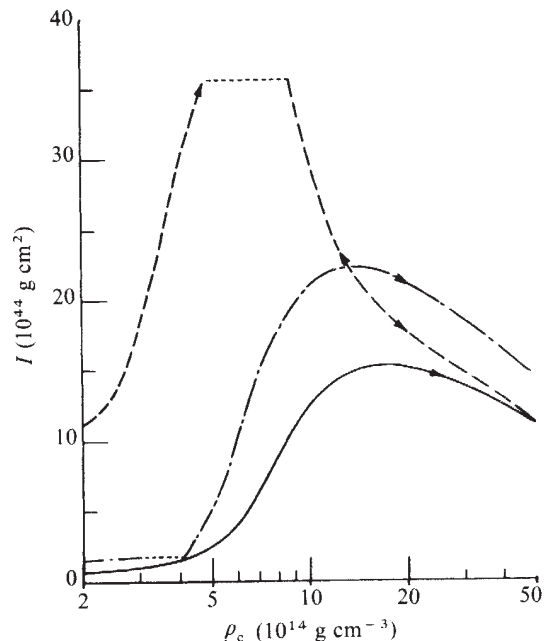


Fig. 3 Stellar surface redshift z plotted against central density ρ_c for the three cases shown in Fig. 2, using the same notation.

the Oppenheimer-Volkoff equation of hydrostatic equilibrium²² to calculate the masses, surface redshifts, and moments of inertia of hydrostatic, spherically symmetrical, nonrotating collapsed stars in the context of the theory of general relativity. The results of some of these calculations are summarised in Table 1 and Figs 2-4.

For all these cases, we found that the maximum stable mass of a collapsed star lies in the range $1.6-2.3 M_\odot$ (see Table 1 and Fig. 2); the value of the maximum mass tends to increase with decreasing ρ_0 and with increasing stiffness of the nuclear-matter equation of state^{5,6,15-17,23}. With the class of quark-matter models that we have considered, an appreciably lower maximum mass for either quark stars or neutron stars could only be obtained by using a softer nuclear-matter equation of state, and an appreciably higher mass could only be obtained by using a harder nuclear-matter equation of state or by requiring that $\rho_q \leq \rho_n$. Thus, the range of allowable stellar masses that we obtain is comparable to that obtained in conventional nuclear-matter calculations of neutron-star struc-

Fig. 4 Stellar moment of inertia I plotted against central density ρ_c for the three cases shown in Fig. 2, using the same notation.



ture¹⁵⁻¹⁷. Moreover, the ranges of allowable surface redshifts (see Table 1 and Fig. 3) and moments of inertia (see Table 1 and Fig. 4) are quite wide at any given stellar mass, and are again comparable to the allowable ranges of these parameters predicted by conventional neutron-star models (compare with ref. 14).

We conclude that stable collapsed stars containing quark matter are entirely possible; and at least one class of physically plausible models for quark matter predicts stable quark stars with masses, surface redshifts and moments of inertia that are substantially the same as those predicted by presently conventional neutron-star models. We note, however, that there may be other important differences between quark stars and neutron stars. For example, a phase transition from nuclear matter to quark matter may exclude the possibility of a neutron solid²⁴⁻²⁶ in the core of a collapsed star. In this case, the giant glitches in the pulse period of the Vela pulsar²⁷ could not be the result of 'corequakes' in a neutron solid²⁸, and an alternative explanation for this phenomenon would be required. It is interesting to speculate that the giant glitches are caused by a metastability in the phase transition in a narrow range of densities near ρ_q , which results in sudden small changes in the moment of inertia of the quark star.

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Apparent superluminal expansion velocities in the dipole magnetic field model

VERY long baseline interferometry (VLBI) observations of compact radio sources have shown that the components of some radio sources seem to move apart with relative velocities more than twice the speed of light. These observations and many interpretations have been reviewed by Cohen *et al.*¹ and Blanford *et al.*². Sanders has presented a model for compact extragalactic radio sources in which apparent superluminal expansion arises naturally. In this model, radiating particles move at relativistic velocities along dipole magnetic field lines. Sanders discusses the relationship of this proposal to other

explanations of superluminal velocities in compact radio sources. However, he incorrectly evaluated the consequences of the dipole field model, obtaining results both for the magnitude of the expansion velocity and its time dependence that are in conflict with observations. We present here the valid geometrical consequences of the model, show that they agree with available observations, and make some additional predictions. A detailed derivation of the equations used here, the plasma-physics aspects of the model, and the possible relation to extended radio sources will be discussed in detail elsewhere.

The central object (a quasar or a radio galaxy) is assumed to have a magnetic field, which at the distances of interest can be described as a dipole field (see ref. 3 and Fig. 1). An energetic event in the central object results in the ejection of a burst of charged particles which move along the field lines, forming a (nonspherical) shell or cloud which expands with relativistic velocities. The radiation from these particles is emitted in a narrow cone around the tangent to the local field line. A distant observer can thus see only radiation which is emitted by particles moving in the plane defined by the dipole axis and the line of sight, that is, along a straight line L in the plane of the sky. At any given time, radiation from the front of the expanding cloud is seen from two points (A and B in Fig. 1) on L. For these points, the tangent to the local field line is parallel to the line of sight. The light travel time on the arcs O-A and on A-X

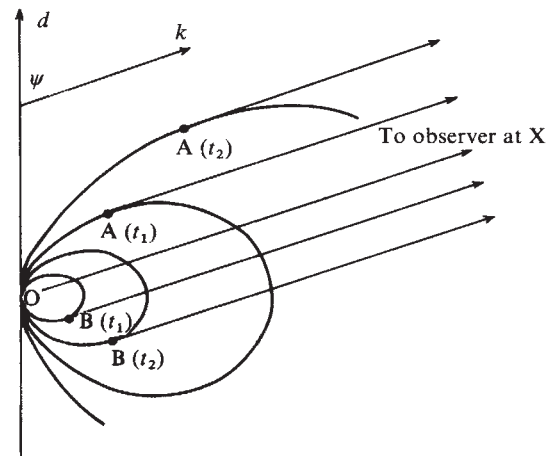


Fig. 1 The geometry of the model. A(t) and B(t) are the points from which radiation reaches the observer at time t. The central object (a quasar or a radio galaxy) is located at O.

(observer located at X) is such that the signal reaches us at time t (the same holds for B). The distance from points A and B to the line O-X changes with velocities V_1 and V_2 , respectively, which are the apparent velocities of the two radio sources with respect to the central object as seen by a distant observer in the frame comoving with the object.

We have made simple geometrical assumptions. In particular, we assume that the magnetic field lines remain fixed in space during the observations (and are not affected by the plasma flow). The main predictions are ($V \equiv$ relative velocity $= V_1 + V_2$): (1) $d(PA)/dt = 0$; (2) $d(V/c)/dt = 0$; (3) $V/c \geq V_m/c = 4.4$; (4) $d(PA)/d\lambda = 0$; $d(V/c)/d\lambda = 0$; (5) $V_1 = V_1(V)$; $V_2 = V_2(V)$; (6) probability $(4.4 \leq V/c \leq V_0/c = \cos \psi (V_0/c))$. Where c is the velocity of light, V_m is the minimum possible value of V , λ is the wavelength at which the observations are made, and PA is the position angle of the line connecting the two sources. The predictions, $d(PA)/d\lambda = 0$, and $d(PA)/dt = 0$, result from the fact that radiation can be seen only from a given straight line (L). For the same reason, it is predicted that different bursts in the same source will have the same value of PA (as has been observed for 3C120). The statement that $d(V/c)/dt = 0$ means that the observed rela-

tive-velocity is constant during the expansion; this is also true for V_1 and V_2 separately. This prediction follows because the dipole field configuration does not have a characteristic radius; thus c is the only dimensional quantity in the problem. With the simplest assumptions, it is also predicted that V/c will be the same for different bursts. In the case of 3C120, the velocities of the two bursts observed may be different (although the analysis of the second burst is only preliminary¹). This could indicate that particles of the second burst move on field lines distorted by the first burst (as the distortion preserves the axial symmetry, PA is still expected to be the same) or that the analysis of the observations of the second burst has not properly included the contribution of the first.

The prediction $V/c \geq V_{mc} = 4.4$ means that the apparent relative velocity is always superluminal with a minimum possible value of $4.4c$ which occurs when the angle ψ between the line of sight and dipole axis is $\pi/2$. This result can be used to impose constraints on the Hubble constant, H_0 , and the deceleration parameter, q_0 , which enter into the determination of V from the observed angular velocity of separation. For example, H_0 must be $< 53 \text{ km s}^{-1} \text{ Mpc}^{-1}$ in order to make the inferred value of V/c for 3C273 larger than 4.4 for $q_0 = 0$ and $H < 49 \text{ km s}^{-1} \text{ Mpc}^{-1}$ for $q_0 = 1$. Also $H_0 < 63 \text{ km s}^{-1} \text{ Mpc}^{-1}$ in order to make V/c larger than 4.4 in the first burst from 3C120. Thus, if the dipole-field model is correct, $H_0 \leq 55 \text{ km s}^{-1} \text{ Mpc}^{-1}$. When enough superluminal sources are observed H_0 and q_0 could be determined.

The prediction that $d(\text{PA})/d\lambda = 0$; $d(V/c)/d\lambda = 0$ simply states that both the position angle and velocity are independent of wavelength (which is different from what would be expected on the basis of explanations based on propagation and opacity effects).

The relationships $V_1 = V_1(V)$; $V_2 = V_2(V)$, imply that the individual velocities V_1 and V_2 are specified functions of the relative velocity V (or, equivalently, the angle ψ). The numerical relationship is given in Fig. 2. This prediction should be testable by future observations although no measurements of individual velocities have yet been reported.

The final prediction states that the probability of finding V between V_m and some value $V_0 = \cos \psi (V_0/c)$, where $\psi(V/c)$

is given in Fig. 2. We have assumed that the dipole axes are randomly orientated in space for the sample under consideration. One of the four well-observed sources, 3C279, has an apparent superluminal velocity of $10c$ (ref. 1). One may ask if this value is too large to be considered plausible for the limited observational sample? Using the results shown in Fig. 2, we find that the probability of observing one source with $V/c \geq 10$ in a sample of four sources is ~ 0.4 , which is acceptable.

Sanders³ mistakenly concluded that 4.4 is the maximum value of V/c which can be achieved. Thus, his results apparently conflict with observations for three of the four well-studied sources (3C345, 3C120, and 3C279).

To avoid this problem, Sanders assumed that particles which move on different field lines are ejected at different times. This additional assumption conflicts, in general, with the observationally verified conditions that the relative velocity is constant and that the observed rise time of the burst is short. Sanders also limited his calculations to the special case of $\psi = 90^\circ$ (see Figs 1 and 2) and discussed only predictions (1) and (4) given above.

In principle, it is possible to see two more sources between A and B. These additional sources represent particles that are returning to the origin on the other side of the central object. However, the existence of these additional sources is uncertain as the observer can see them only after two oppositely moving beams have interacted. In any case, these backside points will appear to move with subluminal relative velocities which are always less than $0.1c$.

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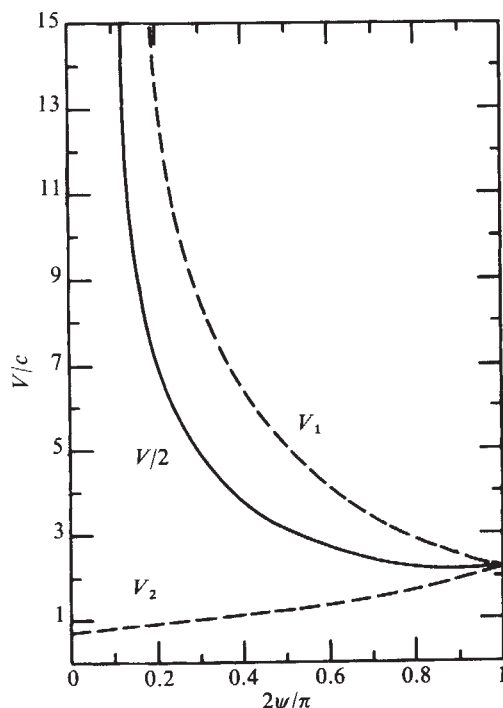
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Fig. 2 The calculated apparent velocities of the faster (V_1), and slower (V_2), components and the presently observed relative velocity (V) as functions of the angle ψ between the dipole axis and the line of sight. A pure dipole field configuration is assumed.



Anomalous acceleration of minor ions in the solar wind

OBSERVATIONS of the solar wind plasma have shown that the bulk speed of helium ions often exceeds that of the protons, provided conditions are such that Coulomb collisions are ineffective in coupling the two species together^{1,2}. We describe here a mechanism which could produce this unexpected effect for any minor ion species, based on the assumption that the sun is a copious emitter of Alfvén waves, as is indeed observed³.

The (nonradial) flow of a minor ion species in a purely radial background solar wind containing a spiral interplanetary magnetic field is governed by the equation⁴:

$$u \frac{du}{dr} = \left[\frac{\Omega r}{U} \left(u \frac{dw}{dr} + \frac{uw}{r} \right) + \frac{w^2}{r} \right] + \nu(U-u) \left[1 + \left(\frac{\Omega r}{U} \right)^2 \right] + F - \frac{1}{\rho} \frac{dp}{dr} + \frac{ZeE}{Am} - \frac{G\mu}{r^2} \quad (1)$$

where u and w are respectively the radial and azimuthal velocity components, Ω is the angular frequency of solar rotation, U is the solar wind (proton) speed, ν is the Coulomb collision frequency between the minor species and protons⁵, F is a wave force⁶, G is the gravitational constant, μ is the solar mass, p and ρ are respectively the partial pressure and density of the

minor species (assumed to have charge Ze and mass Am) and E is the radial (charge-separation) electric field associated with the electron pressure gradient. It can be shown that the latitudinal velocity component v is small and hence that the azimuthal velocity is given by $w = (U - u)\Omega r/U$ (ref. 4). The first term on the right hand side of equation (1) represents effects due to the Sun's rotation and the spiral nature of the interplanetary magnetic field (B_r, B_ϕ), which give rise to a centrifugal force, w^2/r , and a Lorentz force, $(Ze/Am)vB_\phi$.

In the dense plasma near the Sun where collisions with protons are frequent, Coulomb friction accelerates minor ions outwards against the gravitational force, part of which is in any case cancelled by the charge-separation electric field. (The partial pressure gradient of the minor ions should normally have very little effect unless the ion temperature is very high⁷.) Once the effects of gravity are overcome, the wave force, the electric field and the rotational force (given by the first term on the right hand side of equation (1)) assist Coulomb friction in further increasing the minor ion speed so that it becomes comparable with and perhaps even exceeds the solar wind speed. Eventually, well beyond the Alfvén critical point, we expect the acceleration to be small so that the minor ion flow is determined essentially by the first three terms on the right of equation (1) corresponding to the effects of rotation, Coulomb friction, and waves respectively. The force due to Alfvén waves is given by^{4,6}

$$F = \delta \left\{ ((U + V)^2 - u^2) \left(\frac{1}{r} + \frac{1}{2U} \frac{dU}{dr} \right) - 2(U + V)u \frac{d}{dr} \left(\frac{u}{U + V} \right) \right\} + F_{\text{res}} \quad (2)$$

$$\equiv F_w + F_{\text{res}}$$

where V is the Alfvén speed and $\delta = B_w^2/2B^2$ with B the magnetic field strength and B_w the wave magnetic field, determined on the basis of the WKB method⁸ and F_{res} is the contribution to the wave force arising from waves whose Doppler shifted frequency matches the ion gyrofrequency. The wave force on the solar wind, F_p say, is the gradient of the wave pressure $B_w^2/2\mu_0$, which on using the WKB variation of B_w may be written

$$F_p = \delta V^2 \left\{ \frac{3}{r} + 2 \frac{d}{dr} (\log(U + V)) - \frac{1}{2U} \frac{dU}{dr} \right\} \quad (3)$$

Since $F_w \gg F_p$ according as $u \ll U$ resonant wave acceleration; F_{res} , is necessary (in the absence of other effects) to achieve minor ion speeds exceeding the solar wind speed.

However, once $u > U$ has been accomplished by resonant acceleration we observe that the non-resonance wave force tends to bring the minor ion flow into an equilibrium such that $u = U + V$. On the other hand, Coulomb friction and rotational effects tend to bring the ion flow into equilibrium such that $u = U$. In general therefore, we expect the minor flow speed to be 'trapped' in the range $U \leq u \leq U + V$ and hence that the acceleration is indeed small. The 'quasi-equilibrium speed', obtained from putting $du/dr = 0$ in equation (1) in which we use equation (2) for F_w and for simplicity ignore F_{res} and neglect spatial variations of U and $U + V$, is given by,

$$u = u_e = \frac{U}{2(\xi + \delta)} \left[\left\{ \eta^2(1 + \xi)^2 + 4(\xi + \delta) \right\} \times \left(\xi + \eta(1 + \xi) + \delta(M^{-1} + 1)^2 + \frac{rF_e}{U^2} \right)^{1/2} - \eta(1 + \xi) \right] \quad (4)$$

where

$$F_e = -\frac{1}{\rho} \frac{dp}{dr} + \frac{ZeE}{Am} - \frac{G\mu}{r^2}$$

$M = U/V$, $\xi = (\Omega r/U)^2$ is a measure of the strength of rotational effects and $\eta = (\nu r/U)$ is the ratio of the solar wind expansion time to the ion-proton collision time. The relation

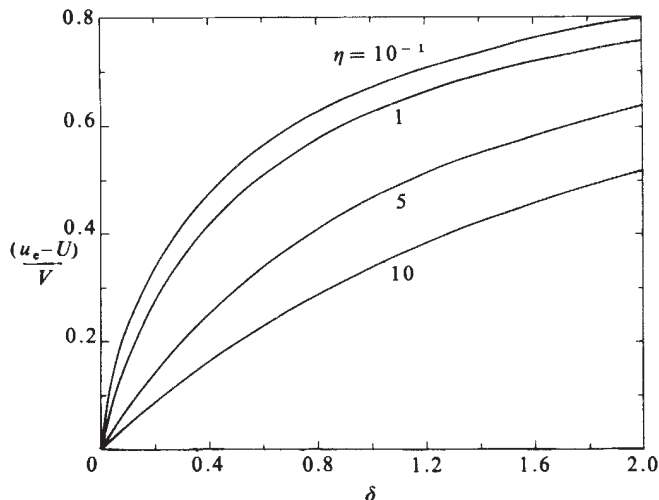


Fig. 1 Variation of the equilibrium differential minor ion speed with wave energy flux (δ) for various values of the ratio η = (solar wind expansion time/Coulomb collision time) for typical conditions at $r = 1$ AU. $\xi = 1$, $M = 8$.

(4) is shown in Fig. 1 for the case $\xi = 1$ and $M = 8$ corresponding to conditions typically observed at $r = 1$ AU for which we can neglect rF_e/U^2 which is $O(1/M^2)$.

This result is in good qualitative agreement with the findings of Neugebauer² to the effect that the differential flow speed between helium ions and protons in the solar wind is controlled by the parameter η , as is the temperature difference. We predict on this basis that the difference in flow speeds decreases with increasing heliocentric distance (increasing ξ) and, provided there is sufficient wave energy flux and Coulomb friction is ineffective, that it increases with decreasing heliocentric distance (increasing V and decreasing ξ). This is made evident by putting $\eta = 0$ in equation (3), which yields $u_e \rightarrow U$ as $\xi/\delta \rightarrow \infty$ and $u_e \rightarrow U + V$ as $\xi/\delta \rightarrow 0$.

We stress that the 'quasi-equilibrium speed' given by equation (4) is qualitative because resonant wave acceleration has been neglected and the effects of the waves on the solar wind are not dealt with consistently.

In general ν and η are functions of $(U - u)$ and of the proton and minor ion temperatures and it is possible for more than one equilibrium speed to exist. For this to be so, it is necessary that the thermal speeds of the protons and minor ions should be small compared with V , in which case two stable equilibrium speeds may exist, one such that $u_e \approx U$ and the other such that $u_e \approx U + V$.

We are now performing numerical calculations to determine the combinations of wave energy and solar wind proton fluxes necessary to produce minor ion bulk speeds exceeding the proton bulk speed in accordance with observations and to determine the conditions under which more than one equilibrium speed can occur.

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Natural enrichment of elements in snow

NATURAL enrichment has been reported for Zn, Cd, Pb and S in Greenland snow dated between AD 1170 and the 1800s using Al as the reference element for the Earth's crust¹. In other studies, not on the determination of enrichment, other elements were analysed in polar ice which had been deposited before man's disturbance of the natural atmospheric composition^{2,3}. Data on Al or other crustal reference elements such as Si and Sc were unavailable for these latter ices. However, their Mn content had been determined, and we show here by the analysis of recent precipitation in the Alaskan Arctic that Mn also serves as an indicator of the weathered lithospheric dust component. This enables the data to be re-examined to assess the degree of enrichment of yet another group of elements: Hg, As, Sb, Se and Cu. This evaluation indicates that the atmosphere is naturally enriched from several units to several orders of magnitude above average crustal abundances, with the elements in the approximate order: $\text{Na} < \text{Cu} \leq \text{Zn} < \text{As} < \text{Sb} \leq \text{Cd} \leq \text{S} < \text{Se} < \text{Hg}$.

The Alaskan snow samples were collected in a north-to-south transect of the North Slope. Barrow, an eskimo community by the Arctic Ocean, is the only inhabited area of significance in the region. These people have a simple lifestyle and would not be expected to seriously affect the chemistry of the immediate environs.

The reference point for the sampling transect was the natural gas well situated several miles south-east of Barrow. On 15 February 1974 seven samples, each about 4 kg, were gathered, and except for a single 56-km span, between 112 and 168 km, all were collected in 28-km increments. The sample sites were lake surfaces reached by aircraft.

One day later, samples were acidified with 10 ml high-purity nitric acid (Ultrex, J. T. Baker) per kg of snow and melted. One month after collection a 1-l aliquot was analysed radiochemically for Hg after neutron activation⁴. In July 1976 another 1- to 2-l aliquot of sample was evaporated at near boiling temperature to a small volume in a quartz still under Class 100 laminar flow conditions. The residual solution (3.0 ml) was neutron irradiated and instrumentally analysed for activation products of Na, Mg, Al, Ca, V and Mn. Zn and Cd were analysed directly by atomic absorption spectrometry.

The elemental concentrations, the nitric acid reagent blank concentration, sample locations and correlation coefficients relative to Mn are shown in Table 1. The error for the elements determined by instrumental neutron activation represent the statistical counting error, and those for Zn and Cd are the deviations from the average of duplicate measurements. In the Hg assay the error is about 15% for values above the detection limit ($0.005 \mu\text{g l}^{-1}$). Manipulations involved in the evaporation step have been shown¹ to be noncontaminating.

Variations in the concentration of elements occur from site to site accompanied by a definite correlation between Mn and Ca, Mg, Mn, Al and V (Table 1). The pattern for sodium suggests a maritime effect as the concentration decreases with the distance from the coast. The patterns are dissimilar for Zn, Cd and Hg, although elevated values occurred at the first collection site for Cd and Hg.

A crustal enrichment factor (EF), defined as the concentration ratio of an element to Mn in the sample compared to this ratio in crustal material, was calculated (Table 2) on the basis of the average crustal abundances compiled by Taylor⁵. For Mn, Ca, Al and V, whose spatial deposition patterns were comparable, the EFs ranged close to unity. This relationship was taken as evidence of their common lithospheric origin. On the other hand, the enrichment factors for Zn, Cd and Hg suggest significant input from other sources. The EF for sodium reflects the marine aerosol contribution from the adjoining Arctic Ocean. For each sample, the quantity of sodium present above that to be expected from crustal contribution was calculated. With this value the elements were normalised for the sea-salt component. This correction reduced the EF for Mg from 2.5 to 1.8, but the values for the other elements were unaffected. Thus, the enrichments observed seem to be unrelated to a maritime effect.

EFs calculated from data based on Mn content and acquired from snows deposited before this century at the New Byrd Station, Antarctic², Camp Century^{2,3}, Dye-3 (ref. 3), and Milcent, Greenland¹ are also shown in Table 2. Also an 800 BC Camp Century and AD 1724 New Byrd Station sample were analysed for their Cu and Mn content by a method described elsewhere⁶. These samples contained 420 and 79 ng l^{-1} Mn, and 840 and 268 ng l^{-1} Cu, respectively. From these values additional EFs were estimated. The As and Sb EFs at Dye-3 were computed with an average Mn value ($250 \pm 170 \text{ ng l}^{-1}$) derived from 19 seasonal precipitations over the period 1967–71 (ref. 3). As data from Greenland indicate that the Mn concentration in contemporary snow is comparable to that of older deposits³ this approach provides a reasonable estimate for the EFs of these two elements. For consistency the EFs previously calculated for Milcent samples were recalculated with Mn rather than Al as the reference element. The recomputed values are greater by 10%.

With a few exceptions there is general agreement for the EFs between the polar and the Alaskan locations. Al and V are unenriched at Barrow and Milcent, and indicate a relatively uniform composition of windborne lithospheric component in these two widely separated northern sites. The EFs of Na, Zn and Cd also agree between these two locations as well as with other Greenland sites where data are available; however, in absolute quantity both the crustal component and the enriched elements are greater in Alaska. At Camp Century no significant difference in enrichment of S, Se and Hg was found between the older 800 BC deposit and more recent ice deposits of the 1800s. The EFs for Antarctic ice were usually substan-

Table 1 Concentration of elements in nitric acid reagent and at various locations from the Barrow Gas Well and correlation with Mn

Element ($\mu\text{g l}^{-1}$)	10-ml nitric acid	Distance from Gas Well (km)							Coefficient of correlation with Mn
		28	56	84	112	168	196	224	
Na	0.4	152 ± 4	149 ± 4	136 ± 3	86 ± 1	64 ± 3	73 ± 3	68 ± 3	0.59
Ca	0.2	75 ± 9	138 ± 11	86 ± 9	35 ± 2	45 ± 8	45 ± 8	6 ± 10	0.93
Al	0.09	30.1 ± 0.3	119.5 ± 0.3	28.3 ± 0.3	15.2 ± 0.1	22.4 ± 0.3	21.3 ± 0.3	39.8 ± 0.4	0.84
V	< 0.001	0.098 ± 0.004	0.255 ± 0.008	0.078 ± 0.004	0.042 ± 0.001	0.095 ± 0.005	0.072 ± 0.005	0.128 ± 0.006	0.80
Mg	0.03	23 ± 3	104 ± 5	37 ± 1	30 ± 1	51 ± 5	55 ± 5	51 ± 5	0.75
Mn	0.002	0.59 ± 0.13	2.22 ± 0.09	1.61 ± 0.08	0.30 ± 0.01	0.63 ± 0.06	0.72 ± 0.06	0.88 ± 0.07	—
Zn	< 0.01	1.07 ± 0.06	0.92 ± 0.02	0.76 ± 0.02	1.15 ± 0.02	0.72 ± 0.02	1.57 ± 0.02	1.67 ± 0.02	0.33
Cd	< 0.01	0.22 ± 0.03	0.02 ± 0.00	0.02 ± 0.00	0.06 ± 0.01	0.02 ± 0.00	0.08 ± 0.01	0.08 ± 0.01	-0.45
Hg	< 0.005	0.026	≤ 0.005	0.014	0.009	≤ 0.005	≤ 0.005	≤ 0.005	-0.16

Table 2 Enrichment factors of elements in snow from various locations (Mn as reference element)

Element	Alaska*	Greenland			Antarctic	
	North Slope Age: 1974	Camp Century 800 BC	1800s	Dye-3 ⁽⁴⁾ ≤ 1900	Milcent ⁽¹⁾ 1170–1885/86	New Byrd Station 1724
Na	5.6				4.4	
Mg	2.5					
Al	0.5				1.1	
S		480 ⁽²⁾	462 ⁽²⁾	271	1,555	1,218 ⁽²⁾
Ca	1.8					
V	0.9				≤ 1.8	
Cu		34	18*	11		59*
Zn	23			20	16	
As				65		
Se		1,132 ⁽²⁾	783 ⁽²⁾			3,391 ⁽²⁾
Cd	518		306 ⁽⁴⁾	646	489	
Sb				342		
Hg	≤ 212	1,720 ⁽³⁾	2,529 ⁽³⁾	2,470		11,273 ⁽³⁾

* This work

tially greater than for Greenland; this difference is attributable to the relatively constant concentration of the enriched elements in the deposits of these polar regions; the dust component is 5–10 times lower in the Southern Hemisphere⁷.

Thus, enrichments in the pre-industrial ice deposits extend over a range of units and tens of units for Na, Cu, Zn and As to several orders of magnitude for Cd, Sb, S, Se and Hg.

To explain these enrichments it should be noted that with the exception of Cu, and discounting the maritime-derived Na, these elements and some of their compounds are relatively volatile. Clearly, the temperatures associated with volcanism would be high enough to enhance their atmospheric concentration. Even the more refractory copper has been identified as a component in the emissions from volcanoes⁸. It has also been suggested that the volatilisation at normal temperatures of certain elements and their compounds from surface rocks may make an important contribution to the atmospheric burden⁹. Thus, crustal outgassing has been invoked to account for the level of atmospheric mercury². In addition, the emission into the atmosphere of volatile compounds and particles containing metals from plants has been described for such elements as Se (ref. 10), Zn, Cd and Pb (ref. 11). Obviously, as large areas of the Earth are covered by vegetation, some fraction of the metals present in the atmosphere may come from this source. Atmospheric augmentation also results from the formation of volatile compounds such as H₂S in organic decomposition and perhaps also by the methylations mediated by bacteria.

In conclusion, the atmospheric enrichment of certain elements occurs as a natural process and demands accountability in the interpretation of the chemical composition of modern aerosols.

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Anisotropy of magnetic susceptibility measurements as a coal banding-plane indicator

MAGNETIC susceptibility measurements on coal were probably first made in Europe by Wooster and Wooster¹ and more recently on American coals by Bivins and Ergun². They suggested, based on orthogonal susceptibility measurements of coal cubes, that anthracite coals are anisotropic. But anisotropy of magnetic susceptibility (AMS), a second rank tensor³ which has been well documented^{4,5} has not previously been determined for anthracite and bituminous coals. We report here the results of AMS measurements which were carried out on some near-horizontal anthracite and bituminous coals collected in open cast and deep cast mines located in the South Wales Coal Field. The purpose of these analyses were threefold: to determine (1) if the results are internally consistent; (2) if such AMS measurements on coal are reliable; (3) if the AMS data can be used to solve coal-related geological problems.

AMS measurements are commonly represented for each sample, by an ellipsoid with long (K_a), intermediate (K_b) and short (K_c) orthogonal axes. The contribution of each magnetic grain to the magnetic signal (in samples containing many grains) combines to produce this ellipsoid. Typical results for measurements on two coal seams are reported in Fig. 1. Both seams exhibit AMS K_a and K_b axes which are nearly horizontal, while K_c axes are orientated nearly vertically. The anthracite data show excellent clusters for all AMS axes. In bituminous samples, however, only the K_c axes are well grouped, while the K_a and K_b axes are scattered randomly in a nearly horizontal plane (Fig. 1). The samples shown in Fig. 1 are representative of most AMS measurements on coals so far carried out, yielding nonrandom distributions; some 15% of the coal seams examined, however, yielded random AMS results because the magnetic anisotropy of these samples is below the limits of precision of the torsion magnetometer used for the measurement.

The nearly horizontal K_a and K_b axes exhibited by both seams (Fig. 1), with K_c AMS axes orientated nearly vertically,

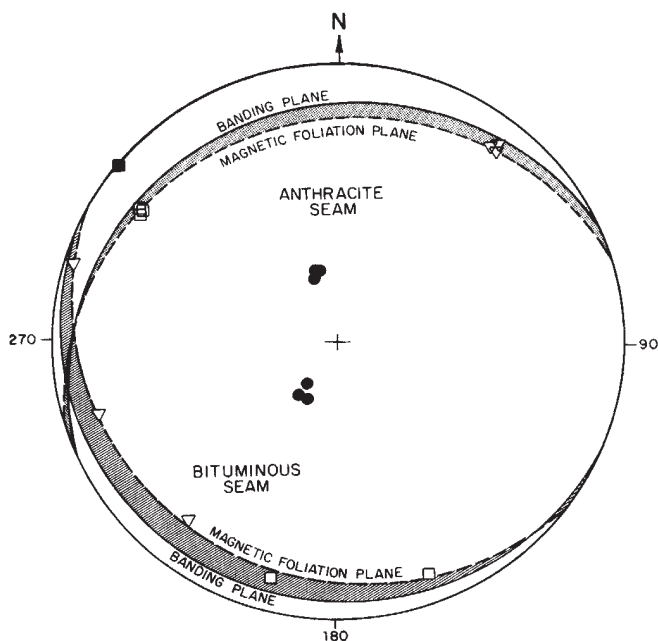


Fig. 1 Equal area projection of AMS data from South Wales Coals. $\square$, K_a axes; ∇ , K_b axes; $\circ$, K_c axes. Open symbols are negative and plotted above the horizon; filled symbols are positive and below the horizon. Solid large circles are the banding planes measured in the field; dashed large circles are the magnetic foliation planes containing the K_a and K_b AMS axes. Shading connects the banding and magnetic foliation planes for each seam. 'N' represents geographic north. The anthracite data are plotted in the upper portion of the diagram, bituminous coal data in the lower portion of the diagram.

are consistent with depositional magnetic fabric⁶. This result is also consistent with coals which were formed, at least in part, by nearly vertical compression⁷ which would align the long magnetic grain axes normal to the direction of maximum compressive stress⁸ (Fig. 1).

The AMS data and the field data are in good agreement, suggesting that the coal banding-plane can be determined, when unknown, from AMS measurements. This provides a means of determining the structural configuration in unexposed coal seams by measuring the AMS in samples taken from several exploratory bore hole cores.

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Evidence for denitrification in aerobic pelagic sediments

PELAGIC sediments cover more than half of the Earth's surface¹, so that the processes which operate within them are potentially very significant in global terms. The effects of organic diagenesis on the dissolved oxygen and nitrogen content of water trapped in pelagic sediments are reported here. Because of practical difficulties this aspect of geochemistry has received little attention: although Müller² and Bender *et al.*³ have provided some insight into its complexity and interest. Data is presented here which suggests strongly that oxygenated pelagic sediments supply a globally significant flux of nitrogen (N_2) to the ocean, and that this gas is probably produced by denitrification occurring in microenvironments, such as zooplankton faecal pellets, at or near the sediment–water interface.

Data on the dissolved oxygen and nitrogen content of pelagic sediments have hitherto not been available, although results from some near-shore environments have been reported⁴. There are considerable difficulties in sampling deep-sea sedimentary pore waters for dissolved gases and in analysing the resulting small samples without contamination. Recent improvements in sampling methods^{5–8}, allowing interstitial water to be collected by *in situ* filtration, have considerably reduced these difficulties. Figure 1 shows profiles of oxygen and nitrogen in the pore water of surficial aerobic sediments from stations in the North-east Atlantic taken during cruise 88 of RRS Discovery.

Samples were collected using the I.O.S. *in situ* pore water sampler, similar in concept to that developed at the Woods Hole Oceanographic Institution^{5,8}. The sampler has a stainless steel shaft, 1 m long, fitted with seven filter ports over which filters (0.2 μ m Nuclepore) are secured. After the shaft has penetrated the sediment a hydraulic system powered by a large compression spring causes the pressure in the sample storage coils connected to each port to drop 600 p.s.i. below ambient. Pore water is drawn through the filters into the coils. The PTFE capillary coils previously used have a relatively high gas permeability, and were replaced by stainless steel coils for this study.

After recovery the filters are removed and the samples transferred under slight pressure to syringes fitted to the filter ports. The syringes are immediately cooled to delay bubble formation, and the samples analysed for their gas content as soon as possible, usually within two hours. The sample (0.5 ml) is equilibrated with a small volume of helium in a septum-sealed ampoule, and aliquots of the gas phase are withdrawn for analysis by gas chromatography. A 100- μ l sample is transferred from the ampoule to the chromatograph injection port using a 100- μ l gas tight microsyringe. The transfer is carried out under water to prevent atmospheric contamination. The gases are separated by means of a column of molecular sieve type 5A (40 cm $\times$ 0.6 cm). The overall precision of the analytical method is estimated, from replicates carried through the whole procedure, at 4% standard deviation. Experimental difficulties were experienced at the first station occupied (9624) and these have apparently resulted in a lower precision at this station. The results for all stations taken during the cruise for which bottom water values were obtained are included in Fig. 1, in which the points are plotted relative to the bottom water collected at the same time and in the same way as the pore water samples. Standards were also analysed at each station for absolute calibration: absolute values are not presented here; the absolute accuracy is lower than the precision, because an extra experimental measurement is involved. However, the absolute nitrogen values are consistent with published information^{9,14}. Oxygen values agree satisfactorily with measurements made by Winkler titration of samples obtained by conventional hydrocast at these stations.

The production of dissolved nitrogen gas takes place, according to the usually accepted hypothesis, only at some depth within the sediment where the oxygen level is very low or zero. One would, therefore, expect that the dissolved nitrogen profiles observed in this study would be linear, with a small positive gradient with depth amounting to 1 or 2% m^{-1} . None of the observed nitrogen profiles agree with this picture. In three cases there seems to be an enrichment occurring at, or very close to the sediment–water interface. At each of these stations (9624, 9633, 9636) the bottom water value differs from the mean of the pore water values, treated as a single population, with a probability $>98\%$. A fourth station (9627) shows similar behaviour, except that a single data point, the shallowest, differs sharply from the trend of its fellows. The remaining two stations show apparent nitrogen depletion, followed by enrichment at greater depth; because the bottom-water point is also subject to error, it is difficult to differentiate between this interpretation and the possibility that the data represent a linear increase with depth. In the latter case the slope of the increase would be greater than expected on the basis of the classical diagenetic model mentioned above.

When data seem to contradict an accepted hypothesis, there is the possibility that the data are in error. It is particularly important in the present case to establish that there is no possibility of a systematic error in the determination of the bottom water value, relative to the pore water values, as much

Fig. 1 Profiles of oxygen and nitrogen against depth (cm) below the sediment–water interface. Concentrations are given as percentage enrichment or depletion relative to bottom water samples (●) oxygen; (○) nitrogen. Station positions and depths: 9636, 42°17' N 14°20' W, 5,275 m. 9634, 38°11' N 15°30' W, 5,226 m. 9633, 37°59' N 12°32' W, 4,920 m. 9632, 37°51' N 10°58' W, 5,021 m. 9627, 35°59' N 09°41' W, 4,317 m. 9624, 36°01' N 07°55' W, 1,334 m. Error bars extend one standard deviation of the analytical system on each side of the data points. The bottom water value used as a reference origin is also subject to this error; error bars are omitted from these points only for reasons of clarity.

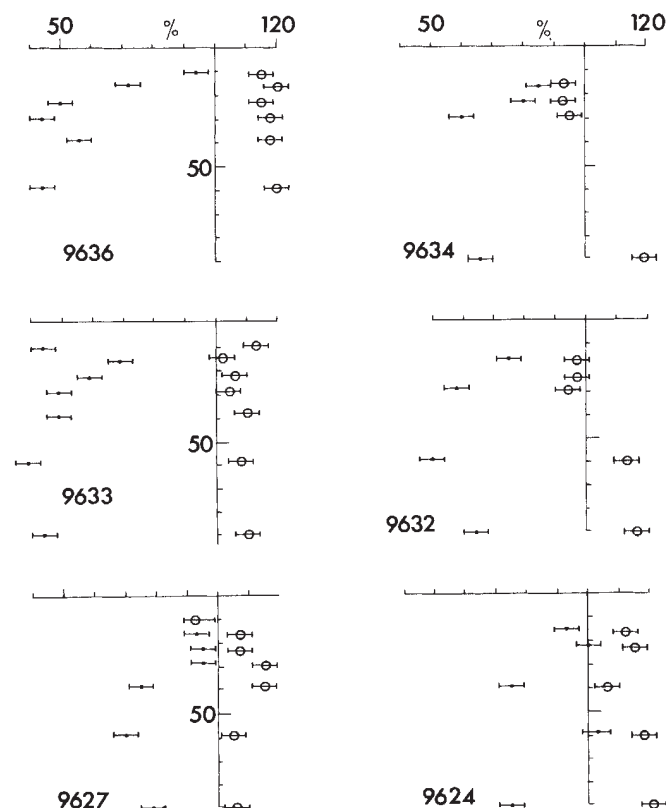


Table 1 Summary of data used to derive oxygen and nitrogen flux estimates

	Oxygen	Nitrogen	Units
Mean gradient	-0.25	0.14	g mol m^{-4}
Maximum gradient	-0.50	0.36	g mol m^{-4}
Minimum gradient	-0.08	-0.12	g mol m^{-4}
Assumed diffusion coefficient	5×10^{-10}	4×10^{-10}	$\text{m}^2 \text{s}^{-1}$
Bottom water gas concentration	0.25	0.60	g mol m^{-3}
Mean flux	1.25×10^{-10}	0.58×10^{-10}	$\text{g mol m}^{-2} \text{s}^{-1}$

Concentration gradients are estimated by drawing a smooth curve through the data points to obtain an estimate of the concentration at 25 cm. The gradient is then calculated on the assumption that it is linear over the top 25 cm interval. This was adopted so as to obtain a conservative estimate of the mean flux. Diffusion coefficients from data given by Horne,¹³ adjusted for the ambient temperature and for tortuosity. Bottom water oxygen concentration values measured by Winkler titration of samples from standard hydrographic casts. Nitrogen value estimated from refs 9 and 14.

of the interpretation hinges on this point. As mentioned above, the sampling system is the same for both pore and bottom waters, the sample being filtered through a 0.2- μm nucleopore filter and stored in a stainless steel capillary during recovery. All subsequent handling and analysis procedures were carried out on the samples in random order, to eliminate any possible systematic effects and no such effects were observed. The findings that dissolved nitrogen is strongly enriched at 1 m depth, and that at four out of the six stations this enrichment occurred at very shallow depths in the sediment column, are, therefore, significant. Dissolved oxygen is depleted between 20 and 60% at 1 m depth, the shape of the curves suggesting maximum activity near the sediment–water interface. These are the experimental findings discussed here. Because of the relatively high random error on individual points the data do not support discussion about apparent deviations from smooth-curve behaviour within the sediment column.

Using the data given in Table 1 it is possible to derive an estimate of the flux of dissolved oxygen into the sediments and of dissolved nitrogen into the overlying seawater. For oxygen the estimates range from 0.45×10^{-10} to $2.7 \times 10^{-10} \text{ g mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$. Values for the oxygen uptake of deep sea sediments of $4.5 \times 10^{-8} \text{ g mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$ have been obtained by *in situ* bell jar experiments¹⁰. These latter experiments, however, included the respiration of organisms living at the sediment surface. When these were poisoned with formalin the oxygen uptake fell to less than 1% of the unpoisoned value (that is, $<4.5 \times 10^{-10} \text{ g mol O}_2 \text{ m}^{-2} \text{ s}^{-1}$) which falls very close to the range calculated here is the oxygen demand of the biomass within the sediment itself; the shape of the curves is that expected from the reported distribution of bacteria with depth. The main geochemical interest of these data is the information this gives on the progress of organic diagenesis within the sedimentary column. Without direct measurements the oxygen levels can only be inferred, rather unsatisfactorily, from measurements of other species. Especially significant are those taking part in anaerobic life processes, such as nitrate and sulphate.

Recently published measurements³ of this sort have suggested that denitrification may be important in pelagic sediments. The observations reported here strongly support this hypothesis. Values for the flux of nitrogen from the sediments, calculated from the profiles given in Fig. 1, range up to $1.6 \times 10^{-10} \text{ g mol N}_2 \text{ m}^{-2} \text{ s}^{-1}$ (station 9636), with a mean of $0.6 \times 10^{-10} \text{ g mol N}_2 \text{ m}^{-2} \text{ s}^{-1}$. Bender *et al.*³ calculated values of 0.4×10^{-10} to $0.95 \times 10^{-10} \text{ g mol N}_2 \text{ m}^{-2} \text{ s}^{-1}$ from their mean data, the variation arising from uncertainties in the stoichiometry of the reactions involved. The coincidence of the results obtained by these two very different methods is striking, and rather surprising. The productivity in the region studied by Bender *et al.* is higher than that in the area of the present study: nitrate

profiles from the present stations (see, for example, Table 2) show that the shape and absolute values in the top 100 cm at these stations correspond approximately to the top 10 cm of the profiles of Bender *et al.* Consequently, their calculated flux at these stations would be much lower than that calculated here directly from the nitrogen concentration profiles. This is because the shape of the nitrogen profiles (particularly at stations 9627, 9633 and 9636) is not that expected on the hypothesis adopted by Bender *et al.*, that denitrification occurs only after all oxygen has been consumed at depth within the sediment. Instead, it seems that nitrogen is being produced mainly in the topmost, oxygenated, layers of the sediment at these stations. This seems to conflict with the common observation that nitrate levels in reducing environments do not begin to fall until oxygen is exhausted. The conflict can be resolved by the hypothesis, which has been proposed by previous investigators^{11,12}, that denitrification may occur within temporarily isolated microenvironments. In the present case, it seems that zooplankton faecal pellets, which are known to contain denitrifying bacteria¹², might provide a suitable environment for this process to occur.

Table 2 Supporting data from station 9636

Depth below sediment-water interface (cm)	Nitrate ($\mu\text{M l}^{-1}$)	Alkalinity (mEq l^{-1})	Total CO_2 (mmol l^{-1})
0	18.8	2.30	2.2
11	—	2.37	2.1
16	26.5	2.47	2.3
23	28.5	2.85	2.4
29	39.7	—	2.5
39	34.1	2.87	2.6
59	—	2.88	2.6
90	—	2.75	2.8

This is consistent with normal aerobic decomposition of organic material, with production of nitrate and carbon dioxide. Other profiles examined show similar curves, the detailed shape of which may in part be due to an abrupt change in sediment porosity at about 20 cm. Nitrate was determined by a modification of the method of Armstrong¹⁶. Alkalinity was determined by microtitration and total carbon dioxide by gas chromatography.

It is important that the enrichments of dissolved nitrogen reported here are greater than would be expected if the only source of nitrogen was dissolved nitrate supplied from the bottom waters. Nitrate is enriched in surficial oxygenated sediments of the type investigated here by its production during aerobic diagenesis of detrital organic nitrogen. Such enrichment is observed in these samples, but the values are not sufficient to support the largest dissolved nitrogen enrichments observed, and the distribution with depth does not suggest that the large dissolved nitrogen enrichments at shallow depths observed at some stations can be derived from this source. However, the flux of nitrogen (as organic and inorganic species) within faecal pellets is more than sufficient to support the observed dissolved nitrogen enrichments. Bishop *et al.*¹⁵ have recently reported a flux of 7×10^{-9} mol organic carbon $\text{m}^{-2} \text{s}^{-1}$ to the sediments as faecal pellets. The C:N atomic ratio in the organic fraction is given as about 10:1, implying a flux of nitrogen in this form equivalent to 3.5×10^{-10} mol $\text{N}_2 \text{m}^{-2} \text{s}^{-1}$. Thus, <20% of the organic nitrogen delivered to the sediments in faecal pellets would be adequate to balance the mean nitrogen gas flux of 6×10^{-11} mol $\text{N}_2 \text{m}^{-2} \text{s}^{-1}$ from the sediments calculated above from the gas data. The mechanism may operate in the following manner. Oxygen diffuses into the pellets, so that an outer oxic zone surrounds an inner anoxic core. Organic nitrogen is oxidised to nitrate in the outer zone, and this nitrate is available for denitrification within the core, the product nitrogen gas then diffusing outwards. This process

would be expected to continue until either the peritrophic membrane disintegrated or until oxygen consumption rate fell below the level necessary to sustain the anoxic core.

The data presented here strongly imply that the surficial pelagic sediments are a geochemically significant site for denitrification. Söderlund and Svensson¹³ estimate a total global marine denitrification rate of 2.9 tonnes s^{-1} . The flux from pelagic sediments, calculated on the somewhat tenuous assumption that the mean nitrogen gradient reported here is typical of all pelagic sediments, is about 0.5 tonnes s^{-1} of nitrogen gas. If most faecal pellets break down at, rather than below, the interface this flux could be an underestimate, as the concentration gradient at the interface would be very much higher than the values estimated from the present data. The series of careful measurements of dissolved nitrogen in seawater made by Benson and Parker⁹ provide an upper limit for the total world flux of nitrogen gas to seawater of about 10 tonnes s^{-1} , as they observed conservative behaviour within their accuracy of measurement ($\pm 1\%$).

Studies are in progress to attempt to sample the region of the sediment-water interface more intensively, so as to define the gradient more exactly. It is also possible that experiments could be made to observe the released nitrogen directly, as the largest flux observed (1.5×10^{-10} g mol $\text{m}^{-2} \text{s}^{-1}$) would give measurable increase in the dissolved nitrogen content of water within a suitably designed bell jar in about three months. It would be necessary to inhibit the normal oxygen-consuming metabolism within the jar to prevent anoxic conditions from occurring.

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Neolithic hyperarid period preceded the present climate of the Central Sahel

THE chronology of Pleistocene climatic changes in the Sahara is well known, but that of the Sahel is little understood. Yet, in the Sahel, between the Ferlo Desert (Senegal) and the centre of the Republic of Sudan, there is a nearly continuous belt of fossil dunes. Although their age is unknown¹, they reflect Pleistocene expansions of the Sahara, and thus alternations of wet and dry climates. I have analysed the relict aquatic fauna of the Sahara-Sahel belt, and report here evidence that the present climate of the Central Sahel between the Middle Niger river and the Chad depression was preceded by a hyperarid period.

Table 1 Fauna found at study sites

	Aïr	Adrar (Mauretania)	Ahaggar	Tassili-n-Ajjer	Tibesti	Ennedi
Groups that disappear after droughts and do not recolonise						
Freshwater medusa (Limnocoidea)	—	+	—	—	+	+
Crustacea malacostraca	—	+	+	+	?	+
Fishes: general	—	+	+	+	+	+
<i>Barbus</i>	—	+	+	+	+	+
<i>Clarias</i>	—	+	Tolga	+	+	+
<i>Tilapia</i>	—	+	+	+	+	+
Others	—	—	—	—	+	+
Nile Crocodile	—	Tagant	—	until 1950	—	+
Groups that disappear after droughts but can recolonise						
Odonata:						
<i>Ischnura saharensis</i>	+	+	+	+	?	?
<i>Orthetrum ransonneti</i>	+	—	+	+	+	+
Copepoda:						
<i>Metadiaptomus mauretanicus</i>	+	+	+	+	?	+
<i>Neolovenula alluaudi</i>	+	+	+	+	?	+
<i>Paradiaptomus greeni</i>	+	—	+	+	?	+

In suitable areas of the Sahara-Sahel zone, natural springs (ain, ers) and lakelets (aguelman, guelta) are found. Between 1975 and 1977, I explored 400 such sites, half of them in the Sahel dunes, and half in three mountain areas of the Sahara (the Adrar of Mauretania, the Ahaggar, and the Aïr). The Aïr, which is central to my discussion, is 450 km south-east of the Ahaggar, the geographic centre of the Sahara, and 500 km north-west of the nearest tributary of the river Niger. To botanists, the Aïr is Sudanian^{2,3}, although it lies on the boundary of the Sahara and the Sahel. It has been little studied by zoologists^{4,5} and its aquatic fauna is virtually unknown⁶.

Ten springs and 25 lakelets between the Tamgak fault valley (19°05' N, 8°30' E) and the escarpment of Tiguidit (16°25' N, 9–10° E) were studied. None of them contained fish, and Tuareg of several Aïr tribes associated only the desert lizard, *Varanus griseus*, with the description of fish shown to them. In contrast, mountain areas of the Sahara north of Aïr have relict faunas composed of fish and African invertebrates. The latter are also absent from Aïr (Table 1). However, vertebrate remains have been found in dry lake beds at Adrar Bous, north-eastern Aïr, showing that a fauna consisting of Nile crocodile, hippopotamus and the large predatory fishes, *Lates niloticus* and *Clarias lazera*, lived here until 5,500 yr BP (C yr)^{7,8}. *Lates* is now common in West African rivers such as the Niger, and in the Sahel lakes of the Middle Niger. *Clarias* is found throughout tropical Africa and in upland areas of the Sahara. It can breathe air and survives dry seasons buried in mud. The disappearance of both species (and the other animals listed in Table 1) from Aïr can be seen to be the result of a hyperarid period, lasting long enough to dry up previously permanent waters.

To place this hyperarid period between 5,500 yr BP and the present, evidence from Lake Chad is of limited help, although at 5,500 yr BP it was in a phase of shrinking⁹, but at that same time there was extensive aeolian activity in the Haoussa erg, which is now part of the fossil Sahel dunes and separates the Aïr from the River Niger¹⁰. This activity was possible only with an annual precipitation one fifth of today's¹.

There is another clue in the chronology of Saharan rock-paintings. The oldest phase (9,000–5,500 yr BP), during which African game was depicted, was followed by a pastoral phase (5,500–3,000 BP), while the horse appeared about 3,000 yr BP and the camel about the beginning of history¹¹. Cattle frescoes are abundant in the Fezzan, Tassili-n-ajjer, Ahaggar and Tibesti, but not in Aïr, and a skeleton of domestic *Bos* found at Adrar Bous had an age of 5,760 ± 500 yr BP (ref. 8), intermediate between the old and middle phases. However, thousands of engravings representing game are found in Aïr, especially in canyons facing the Tenere plain. Here, this game phase

is followed closely by the Proto-Tuareg inscription phase in Tifinar language¹².

Representations of horse-drawn wagons are rare in Aïr as well. The prehistoric trans-Saharan horse trade route departing from the Gulf of Sirte branched in the Tassili-n-ajjer, avoiding Aïr¹¹ (Fig. 1). This indicates that the Aïr was not attractive for trade. Furthermore, at the ends of both branches were reliable waters (the River Niger and Lake Chad), suggesting that the southern journey ran through very dry country.

The combined evidence places the extreme drought in the central Sahel at between 5,500 and 3,000 yr BP. At the latter date, hyperaridity also began to occur in the Sahara, destroying its Neolithic civilisations. The current view is that this was due to the southward retreat of the monsoon which continues today¹³. However, this implies that while the Sahara was still relatively humid, the Sahel was even more humid, which is a contradiction of the facts discussed here. This paradox is eliminated by assuming that the monsoon had moved south during 5,500–3,000 yr BP, and that the hyperarid zone shifted to the Sahel, including the Aïr on its northern flank. In modern times, the reverse occurred: the monsoon moved about 500 km north, the hyperarid belt advanced to the Central Sahara and the transition zone between Sahara and Sahel came to be located on the Aïr. This implies that the 'Neolithic' humidity of the Ahaggar-Tassili zone (5,500–3,000 yr BP) was due to northern rains. Pollen analysis has shown that at that time a cold-Mediterranean flora occurred here, relicts of which still exist³. In fact, Saharan mountains have continued to benefit from a residual effect of the Mediterranean climate until the present time and that enabled their aquatic fauna to survive, while the former hyperarid climate of Aïr was not mitigated by that Mediterranean effect, and its original fauna was lost. The contemporary climate of Aïr is arid, moderately influenced by the monsoon. During the Pleistocene, the Oued Azaouak drained the western slopes of the Aïr and Ahaggar and was an important headwater of the Niger. It dried up about 5,500 yr BP, and lately subpluvials were too weak to enable fishes to recolonise the Aïr. The present is a period of further desertification¹⁴, and the Azaouak flows for only a few hours after exceptional rains.

No fish could reach the Aïr from the Ahaggar either. But three non-migratory dragonflies found in Aïr are of Mediterranean (*Ischnura saharensis*) or Mesasiatic origin (*Orthetrum ransonneti*, *Paragomphus sinaiticus*) (Table 1). Residual Asiatic faunas, partly extending to the Ahaggar, are well known from North Africa¹⁵, but their presence in the 'Sudanian' Aïr, especially in the absence of true Sudanian species, is significant. It shows that, even after the hyperarid period had ended, aquatic animals could more easily bridge the gap between Ahaggar and Aïr than between the River Niger and Aïr. This implies that

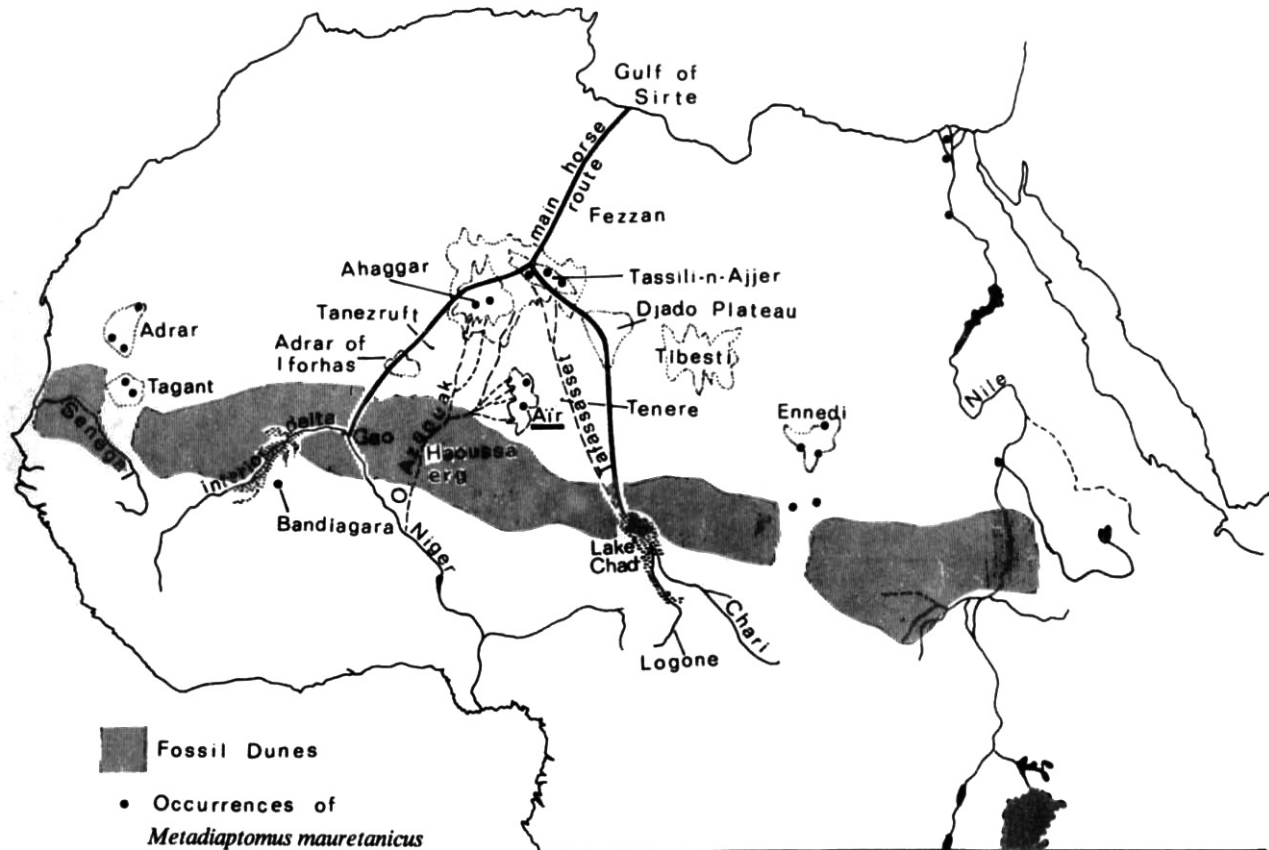


Fig. 1 Sketch-map of the northern half of Africa, showing the main upland areas of the Sahara, the girdle of fossil dunes of the Sahel, and the known distribution of *Metadiaptomus mauretanicus*. Reconstruction of horse-wagon routes, ascribed to Garamantic tribes, modified from H. Schiffrers in ref. 1.

since 3,000 BP, the climate of the Sahel has never been much more humid than today.

An examination of the planktonic copepods of Aïr gueltas substantiates these conclusions. The species found are all typical of the Sahara and of northern or Oriental origin (Table 1), while no representatives of true African genera (*Tropodiaptomus*, *Thermodiaptomus*) occur. However, if relicts found in Sahara mountains bear witness to former pluvial climates, a parallel must also be true, and a past desert phase in the Sahel should not only have left dunes there, but also Saharan relict animals. In March 1976 I found one relict population of *Metadiaptomus mauretanicus* in a pond on the semi-arid plateau of Bandiagara, South of the Niger (Fig. 1), where it co-exists with the African *Thermodiaptomus* and *Tropodiaptomus* and with a Sudanian fish-fauna¹⁶.

In conclusion, it seems that, since 5,500 yr BP, the climate of the Central Sahel was first hyperarid, then arid, and that its aquatic populations are derived from Saharan stocks and not from the Niger River. This supports the thesis¹⁴ that human misuse rather than climatic change is decisive in the present deterioration of the Sahel ecosystem.

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Magnetic field detection is distinct from light detection in the invertebrates *Tenebrio* and *Talitrus*

THE mechanism used by animals for geomagnetic field detection has long been discussed¹. Leask² has proposed that sensitivity to magnetic field might result from polarised light detection through optical pumping into the lowest level triplet state of (say) the rhodopsin molecule that possesses spin. Light detection, he suggests, might be a prerequisite for magnetic field detection and might result in an axial rather than a polar response in the animal. However, a unimodal reaction has been found³ with *Tenebrio molitor* L., the flour-beetle, in a horizontally directed magnetic field and a light field without directional features. This result was obtained when *Tenebrio* had been allowed to associate the geomagnetic field with the directional properties of the anisotropic light field in the culture container. The preferential direction in the horizontally directed magnetic field could be predicted from the 'dark direction' and from the relative humidity in the container (ref. 3, expt 43, page 429). I report here that *Tenebrio* and *Talitrus saltator* Mont., the sandhopper, can orientate in the Earth's magnetic field in complete darkness, and that in *Tenebrio* this orientation is

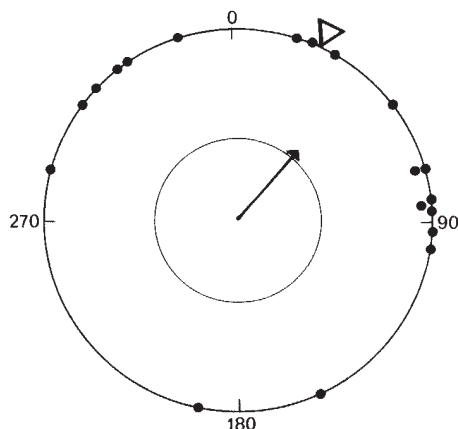


Fig. 1 Distribution of direction choices of *Tenebrio* imagines tested in complete darkness in the local geomagnetic field. The animals were introduced into the set-up while the light was on. ∇ : Direction predicted from the dark direction and relative humidity (RH) in the culture container. $\bullet$: Direction of one trace. Outer circle: circle with radius $r = 1.0$. Direction of arrow represents the observed mean direction ($\theta = 40^\circ$), its length the statistic of concentration ($r = 0.43$; $P < 0.05$). The inner circle indicates the 5% significance level (Rayleigh test⁴). The reactions are clustered around the predicted direction (V-test: $P < 0.005$). Magnetic north (mN) is at zero degrees in all figures, unless otherwise indicated.

essentially polar, not axial. These results are evidence that these two invertebrates do not require light for the detection of the magnetic field.

The first experiment was performed with *Tenebrio*, cultured in an anisotropic light field. From our previous results³ on the magnetomenotaxis of *Tenebrio* orientation was expected in a direction 26° east of magnetic north, the 'dark direction' in the culture container. Two days before the experiment the rearing box was placed in darkness. The beetles were tested for directional preference after extracting them from the dark box and placing them on a platform covered with starch. After one animal had been placed on the platform it was covered by an opaque box (diameter 3 cm) and the light in the room was switched off.

After 5 min the box was removed. The trail left by the beetle in the starch during the following 5 min was copied on graph paper. The direction of the trail was determined as the direction of the radius drawn from the centre of the platform parallel to the straight line between the starting point of the trail at the wall of the opaque box and the trail's endpoint at the border of the platform. The distribution of trail directions is not uniform (Fig. 1) and, moreover, their mean compares with the predicted direction (V-test⁴).

Because in the first experiment the beetles were introduced to the set-up when the light was on, the results might not be convincing. Therefore, in the second experiment, two tests were performed in total darkness, following a suggestion of Leask⁶. Thirty minutes before the test was performed the beetles were placed in complete darkness. In darkness they were put on the platform under an opaque box which was removed after 5 min. The platform was completely surrounded by 12 numbered plastic cups so that, when a beetle arrived at the edge of the platform, it would fall into one of the cups. Each time this occurred the light was switched on and the number of the cup containing the beetle was recorded. Hence a frequency distribution in 12 direction classes was obtained. And each time the platform was rotated 72° (compare ref. 7), and the light switched off before a new beetle was introduced.

Because the dark direction in the culture container was 330° , this is the expected direction for the magnetotactic reaction at the moderate relative humidity in this container (RH = 65%).

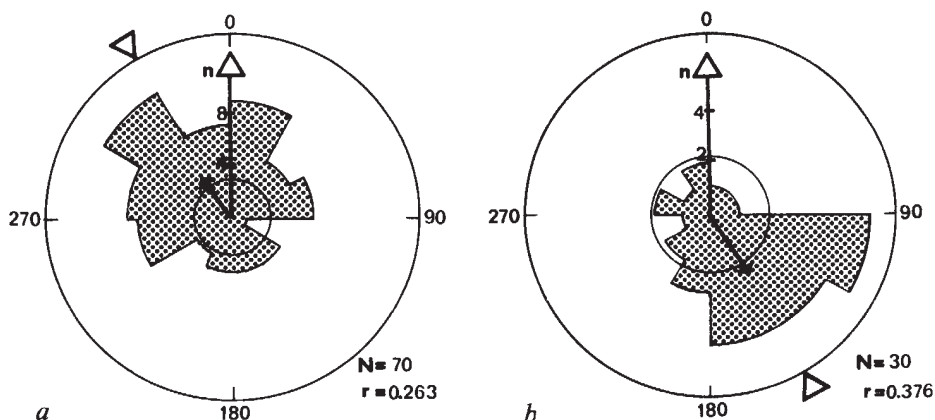
The frequency distribution of direction choices was unimodal and significantly different from uniform (Fig. 2a, Rayleigh test). The direction choices are clustered around the predicted direction (V-test, $P < 0.01$). The beetles used in the test were then placed overnight on a layer of very wet cotton wool in a small glass jar. The RH in the jar rose to 90%. In such conditions the sign of the magnetomenotactic reaction changes, just as it does when *Tenebrio* is kept in very dry conditions³. Hence the expected direction of the reaction becomes 150° . The test was repeated with 30 specimens. Again the distribution was unimodal, significantly different from uniform (Fig. 2b, Rayleigh test) and clustering around the mean direction was evident (V-test, $P < 0.01$).

It is concluded that *Tenebrio* displays a nonvisual orientation in total darkness which has all the characteristics of the magnetomenotaxis observed earlier³ in an isotropic light field.

The third experiment was performed with *Talitrus*. A different set-up was used consisting essentially of a hemispherical bowl in total darkness, placed in a set of Helmholtz-coils. After 5 min in the local geomagnetic field ($H = 0.47$ Oe) the animals and a compass card below the bowl were flash-photographed together. During the subsequent darkness the magnetic field was rotated 90° in an anticlockwise direction without alteration of the strength or inclination of the magnetic field. After another 5 min of total darkness the group of sandhoppers was flash-photographed again. The photographs of six groups of nine or ten animals were analysed. From the photographs two frequency distributions were obtained, one for the animals in the local geomagnetic field (Fig. 3a) and one for those in the rotated magnetic field (Fig. 3b). Both distributions seemed to be bimodal. Such distributions can be analysed by 'doubling the angles'^{4,9}. The frequency distributions obtained in this way are given in Fig. 3c and d.

Bimodal distributions can be considered as being mixtures of von Mises distributions. Mardia⁹ gives a procedure for estimat-

Fig. 2 Frequency distributions of direction choices of *Tenebrio* (imagines) tested in the local geomagnetic field in complete darkness. The animals were introduced into the set-up in darkness. θ : Observed mean direction of the frequency distribution. r : Statistic of concentration in the Rayleigh test. n : No. of observations in each sector which are proportional to length of sector radius. N : Total no. of observations. a, *Tenebrio* taken from culture container with RH = 65%; $r = 0.263$; $P < 0.01$. b, *Tenebrio* taken from culture container with RH = 90%; $r = 0.376$; $P = 0.01$. Other indications as in Fig. 1.



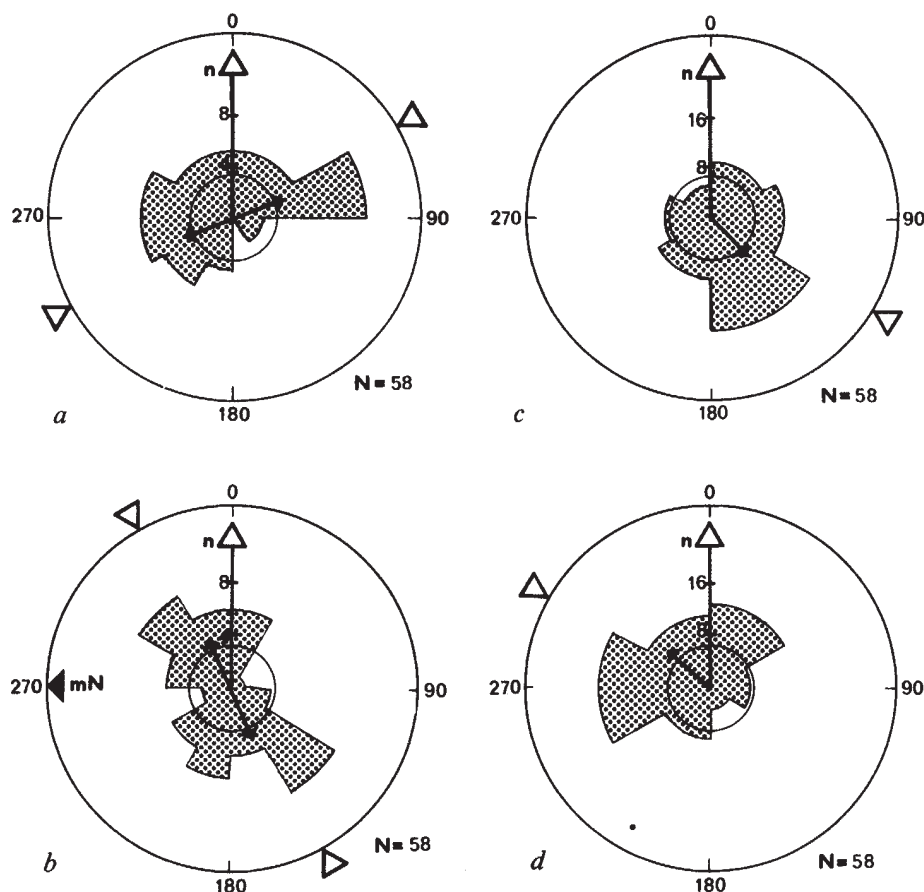


Fig. 3 Orientation of *Talitrus* in the local geomagnetic field (*a* and *c*) and in an artificial field in which magnetic north is at 90° (*b* and *d*). The animals were introduced into the set-up while the light was on, but the magnetic field was rotated during darkness. After rotation of the magnetic field no light was provided before a flash photograph was taken. *a* and *b* show the observed distributions; *c* and *d* show the frequency distributions obtained by 'doubling of the angles'. Observed symmetry-axes ($\leftrightarrow$) in (*a*) and (*b*) are computed from the 'doubled' frequency distributions in (*c*) and (*d*). Other indications as in Figs 1 and 2.

ing the proportion (λ) of animals that have moved in either of two opposite directions. If both modes are equally pronounced, then $\lambda = \frac{1}{2}$. In a uniform distribution also, $\lambda = \frac{1}{2}$. Doubling the angles in a uniform distribution will not significantly affect the value of λ , but in a true bimodal distribution λ will shift to a value $\lambda > \frac{1}{2}$. Both modes will be significantly different if $\lambda > \frac{1}{2}[1 + \sqrt{(\chi^2_{\phi-1}/N)}] = CV(\lambda_N)$, where N is the total number of observations and $CV(\lambda_N)$ denotes the critical value of λ . When $N = 58$, $CV(\lambda_N) = 0.65$. I have accepted bimodality if the three following requirements are met: (1) before doubling of the angles $\lambda \leq CV(\lambda_N)$; (2) after this procedure $\lambda > CV(\lambda_N)$; (3) skewness (g), determined according to Batschelet⁴, is negligible. Table 1 summarises the relevant data. I conclude that both distributions are bimodal.

The statistical tests were performed on the distributions obtained by doubling of the angles^{4,9}. The distributions from both tests were nonuniform (see Fig. 3) and significantly different (χ^2 test⁴, $P < 0.02$) from each other. The variances of both distributions were equal (two-sample test of concentration statistics according to equation 6.3.37 of ref. 9, ($P > 0.37$)). Hence the mean directions were significantly different. The mean direction of the distribution obtained before the rotation of the magnetic field was 136° (Fig. 3c). After halving this angle, this compared very well with the ecologically important direction perpendicular to the direction of the coast of origin (Fig. 3a, V -test: $0.005 > P > 0.001$). The mean direction after the rotation of the magnetic north was 312° (Fig. 3d). The half angle, 156° , compared with the expected direction parallel to the coast of origin (Fig. 3b; V -test: $0.001 > P > 0.0001$). It is concluded that *Talitrus* is able to orientate in an ecologically important direction using magnetic field information; this is at variance with the results of Ercolini and Scapini¹⁷ and Scapini

and Ercolini¹⁸. Moreover it is concluded that light is not required for the detection of the magnetic field.

In the fourth experiment, with the set-up and the method described earlier³, *Tenebrio* was tested in an artificial magnetic field ($H = 0.44$ Oe) with zero vertical component which was supplied in an isotropic light field. As before, the beetles had been cultured in an anisotropic light field. Three tests were performed with beetles from containers differing in relative humidity and dark direction.

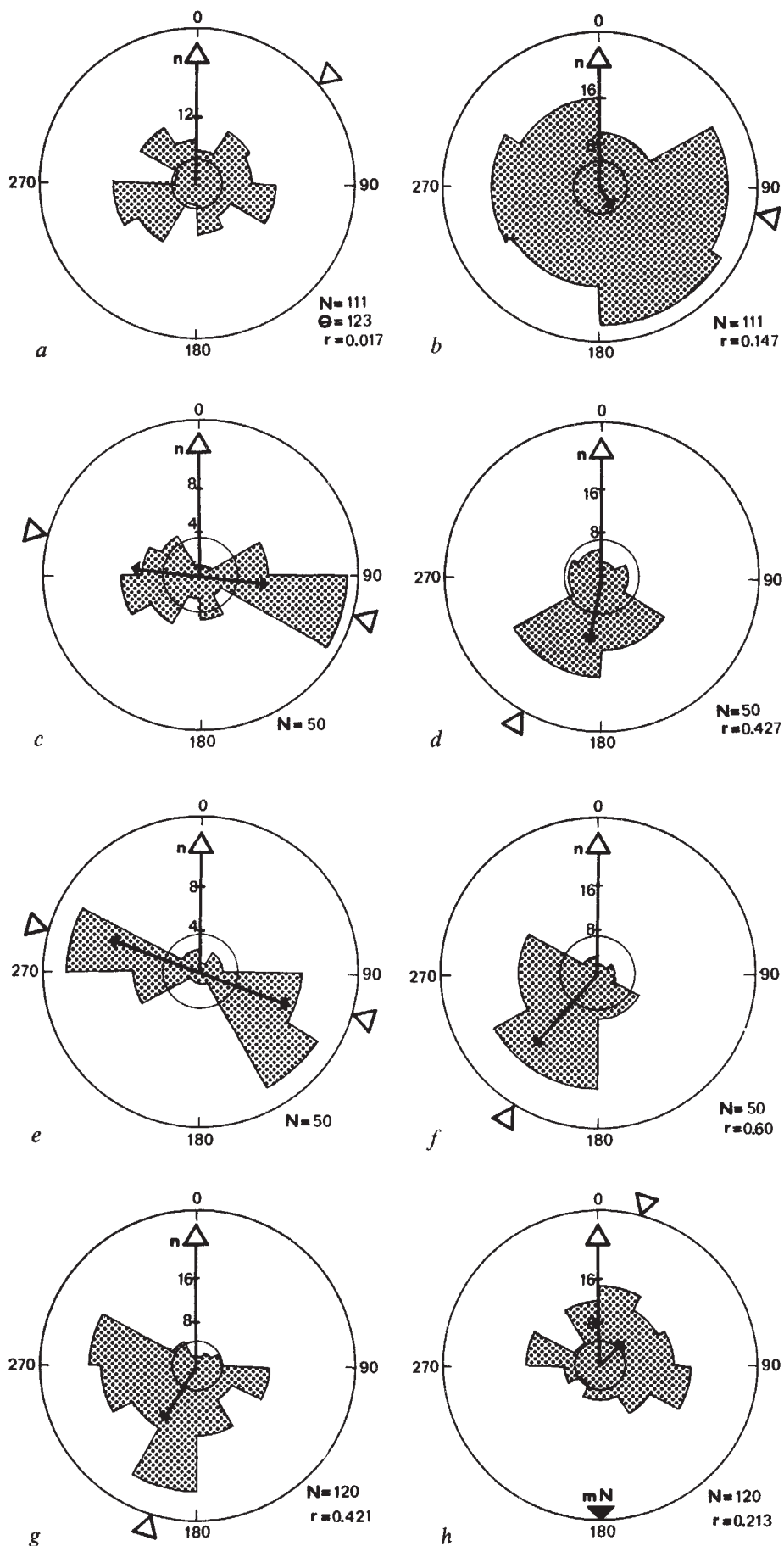
The beetles used in the first test were taken from a container where RH was not under control and varied with the environment. The dark direction was 50° . The frequency distribution obtained was uniform (Rayleigh test, $P > 0.10$; Fig. 4a). Because it was known that bimodality might occur, the procedure of doubling of the angles was allowed. However, the new frequency distribution was also uniform (Rayleigh test, $P > 0.10$; Fig. 4b).

In the second test the animals used were from a container with RH = 47% and a dark direction of 105° . Because of the

Table 1 Relative value of the modes and skewness in the frequency distributions of *Talitrus* in the third experiment with $CV(\lambda_{58}) = 0.65$

	Local geomagnetic field		Rotated magnetic field	
	λ	g	λ	g
Original distribution	0.64	0.09	0.58	0.07
Distribution after doubling of the angles	0.83	-0.04	0.77	0.13

Fig. 4 Orientation of *Tenebrio* in the local geomagnetic field and in an artificial magnetic field with zero vertical component (Z). Frequency distributions were obtained in an isotropic light field with *Tenebrio* from containers with different humidity conditions. *a*, Observed and *b*, doubled frequency distribution; humidity not under control; $Z=0$. *c*, Observed and *d*, doubled frequency distribution, RH=47%, local geomagnetic field. *e*, *f*, Corresponding distributions in artificial magnetic field, $Z=0$, and magnetic north at 180° . Note that RH refers to the humidity conditions in the containers where the beetles were kept, but not to the RH during the experiment. *g*, *h*, See text. Other indications as in Figs 1 and 2.



RH value a bimodal reaction was expected. In the dark control test in the local geomagnetic field, a bimodal distribution was found (Fig. 4c). After doubling of the angles this was significantly different from uniform (Rayleigh test, $P < 0.0001$; Fig. 4d) and the mean direction compared with the expected direction (V -test, $P < 0.001$). In the artificial magnetic field with zero vertical component the results were fully comparable. The distribution was again bimodal as expected from previous results³ (Fig. 4e). After doubling of the angles the frequency distribution was significantly different from uniform (Rayleigh test, $P < 0.001$; Fig. 4f) and the mean compared with the predicted direction (V -test, $P < 0.001$). Finally, the 'doubled' frequency distribution from the test in the local magnetic and the artificial magnetic field did not differ significantly ($\chi^2 = 6.42$, $\phi = 3$, $P > 0.05$).

For the third test the beetles were taken from a container in which RH was 69% and the dark direction 195°. From previous results³, a unimodal reaction in that dark direction was expected. In the control test in the local geomagnetic field this was found (V -test: $P < 0.001$; Fig. 4g). The artificial magnetic field with zero vertical component was given a northerly direction which was rotated over 180°. Hence, the expected direction changed from 195° to 15°. In the test the expectation was again confirmed (Fig. 4h; V -test: $P < 0.005$).

Uniformity or bimodality of the frequency distribution of directional choices obtained in a test in a magnetic field with zero vertical component provided with a light field without directional information has been cited repeatedly as evidence for the axial nature of the response to the magnetic field^{2,5}. However, uniform, unimodal and bimodal frequency distributions can be obtained 'at will' in both the local geomagnetic field and the artificial magnetic field with zero vertical component simply by controlling humidity in the culture container (fourth experiment).

The direction of the reaction changes 180° (sign reversal) if the humidity conditions in the culture container are changed (second experiment and ref. 3). This reversal is identical to that observed in the phototaxis of *Tenebrio* kept in the same humidity conditions¹⁹. If the altered humidity level is applied for too short a time, only some of the specimens show the sign reversal³. Then a bimodal frequency distribution is obtained. This indicates that in *Tenebrio* the sign reversal of the reaction, and hence the unimodality or bimodality of the reaction, are the expression of the motivational state rather than an indication of the axial or polar nature of the reaction.

In favourable conditions a unimodal reaction is obtained (Fig. 4g), not only in the geomagnetic field but also in a magnetic field with zero vertical component. Moreover, the direction of that reaction follows the 180° rotation of the magnetic field. This seems to indicate that the animal perceives the polarity of the field.

It is concluded that in *Tenebrio* and *Talitrus* light is not a prerequisite for magnetic field detection and that in *Tenebrio* the reaction is polar rather than axial.

Some peculiarities of the experiments described here should be noted. (1) Both invertebrate species could move freely in the experimental bowl or on the platform. In conditioning experiments with pigeons that failed to show sensitivity to magnetic fields the birds often could not move freely^{10,11}. But when they could, magnetic sensitivity was found¹². In general, sensitivity to the magnetic field seems to coincide with a certain level of rotational activity as follows from experiments with pigeons¹², night migrating birds^{5,13,14}, rays¹⁵, *Tenebrio*³ and *Talitrus* (unpublished). If experimental factors such as the light level decrease the activity level^{2,16}, this might have the same effect as immobilising the animal. (2) The duration of an experiment might be crucial. Often in one individual the direction preference²⁰ as well as the mode of behaviour—circling, repetitive hopping in one direction, as observed in birds⁷—may vary in the course of time. Therefore, in my experiments each animal was in the set-up for only about 10 min. (3) During such a 10-min period, however, some individuals of a species may

show one pattern of behaviour, and other individuals another. I have found that non-hopping *Talitrus* do not show directional preference in the geomagnetic field in an isotropic light field, whereas hopping ones do (Kolmogorov-type test; $P > 0.05$ and $P < 0.005$ respectively; unpublished).

If temporal (2) and/or individual (3) variations in behaviour are neglected, then the results of the analysis suggest that the animals have a weak orientation to the magnetic field; this is what many authors have concluded. In my view, however, these factors must be taken into consideration, and then the orientation to the magnetic field might appear to be quite strong (second experiment).

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Metabolism of the abyssopelagic rattail *Coryphaenoides armatus* measured *in situ*

MEASUREMENTS of the oxygen consumption of two common benthopelagic fishes, *Coryphaenoides acrolepis* and *Eptatretus deani*, made *in situ* at 1,230 m in the San Diego Trough¹ have indicated low respiration rates. The metabolic measurements reported here for the rattail, *Coryphaenoides (Nematonurus) armatus* (Hector), at 3,650 m support the earlier findings of low respiration rates in deep-sea rattails. They also support the physiological axiom that respiration increases as a fractional power of body weight in animals^{2–4}. Both the metabolic rate and chemical composition of *C. armatus* suggest an adaptation to a food-limited deep-sea environment.

The cosmopolitan macrourid, *C. armatus*, lives primarily at depths between 2,000 and 4,000 m in all oceans except the Arctic^{5,6} and can account for as much as 80 % of the benthopelagic fish biomass⁷. Smaller individuals feed entirely on benthic animals but larger individuals feed on pelagic animals^{8,9} and have been caught as high as 685 m above the bottom¹⁰.

I measured the oxygen consumption, excretion and chemical composition of three individuals of *C. armatus* of different size at two stations on the continental rise in the North-West Atlantic. Station ADS (39°05' N, 70°09.8' W), at 2,750 m, was occupied in August 1976 while station DOS2 (38°18.4' N, 69°35.6' W), at 3,650 m was occupied in June 1977. Bottom

Table 1 Respiration, excretion, weight, length and sex of three individual *Coryphaenoides armatus* from stations ADS and DOS2

<i>C. armatus</i>	Depth (m)	Total length (cm)	Wet weight (kg)	Sex	Respiration ml O ₂ per h		Excretion µg-atoms N per h				combined	
					Total	per kg wet wt	ammonia		urea		Total	per kg wet wt
(1)	3,650	48.5	0.5	M	1.9	3.7	0.6	1.2	0.2	0.4	0.8	1.6
(2)	2,753	53.5	0.7	M	2.2	3.1	0.6	0.9	0.3	0.4	0.9	1.3
(3)	3,650	68.5	1.2	F	3.3	2.7	1.3	1.1	1.2	1.0	2.5	2.1

Sample size and mode of measurement prevented replication on each individual animal.

water temperature was 3°C and dissolved oxygen concentration was 6.53 ± 0.11 ml O₂ per l at both stations.

A free vehicle elevator (acoustically controlled) was used to deploy two fish metabolism chambers (FMC) at each station. The submersible DSRV Alvin was used to manipulate the chambers to enclose an individual fish *in situ* in one chamber while the other was closed as a control. FMCs basically consist of a Plexiglas box (50×50×30 cm) with inclined ends. The total volume of the two chambers is 44.4 and 47.6 l. A spring-loaded door with locking latches is situated at the end of each chamber and can be cocked or released by the Alvin manipulator. On top of each chamber are two timed withdrawal syringe systems, a stirring motor and a polarographic oxygen electrode¹¹⁻¹³. The respiration and excretion of each rattail were monitored for periods of 21–22 h. Respiration was measured continuously but excretion rate was based on a two-point determination, with the final sample taken immediately before the elevator was raised. Respiration in the controls was negligible but excretion measurements had to be corrected for the influence of the bait. Bait was enclosed in a nylon mesh bag to lure fish into the traps but prevent feeding.

When measurements were complete, the elevator was recalled acoustically to the surface and recovered. Syringe samples were analysed immediately for ammonia and urea¹⁴ and the fish were frozen for identification and weighing in the laboratory. Displacement volumes of fishes and bait were determined to calculate the final volumes of the FMCs. Bottom water samples taken at the time of FMC closure by Alvin-operated Niskin bottles were analysed for initial concentrations of oxygen and nutrients. Lipid, glycogen and water content were determined for each rattail and the total lipid was fractionated to distinguish the stored energy component¹⁵⁻¹⁷.

Initial respiration rates were elevated 10–15% for the first 20–95 min after capture but levelled off to relatively constant rates. The influence of the initial excitation period, as evidenced by increased respiration rates, on excretion of ammonia and urea is not known and our measurements reflect this probable increase averaged out over the duration of the measurements.

Total respiration of *C. armatus* increased but respiration per g wet weight decreased with increasing size (Table 1), thus exhibiting the same oxygen consumption pattern found in essentially all animals studied²⁻⁴. The relationship between rattail respiration and body weight is best described by the power function $Y = 0.03 W^{0.65}$ ($r^2 = 0.99$) where Y is the oxygen consumption (ml h⁻¹) and W is the wet weight (g). My specimens are intermediate in total length, and probably weight, for the reported size range of *C. armatus* (10–87 cm)^{5,6,9}. The slope of this function is significantly different ($P > 0.01$) from the respiration versus wet weight regression generated for the phylogenetically related shallow water Atlantic cod (*Gadus morhua*) at the same temperature (3°C)¹⁸. However, the slope is comparable with that found for fishes which are adapted to the low food environment of caves¹⁹. The datum on *C. acrolepis* from 1,230 m in the San Diego Trough¹ does not deviate significantly ($P > 0.01$) from my findings for *C. armatus* (in ref. 1 rate of respiration was found to be 2.4 ml O₂ per kg per h).

Rates of excretion (a measure of protein metabolism) of ammonia and urea in *C. armatus* show no definite trend in relation to body size (Table 1). Urea excretion did increase substantially between the two smaller fish (nos 1 and 2) and the larger individual (no. 3). Such a lack of correlation is not surprising because the total nitrogen excretion was probably not measured. Such products as trimethylamine oxide, creatine and creatinine can be important nitrogenous wastes of some shallow water marine fishes^{20,21}. The measured nitrogen excretion varied from 3.2 to 5.9% of the energy expenditure of these fishes as measured by oxygen consumption, assuming a respiratory quotient of 0.75.

There are no significant trends in the chemical composition of *C. armatus* (Table 2). Total lipid and glycogen composition of *C. armatus* does not deviate strikingly from that of the shallow water cod (*Gadus morhua*); however, the neutral lipid fraction (energy storage) is 65% higher in *C. armatus*²²⁻²⁵. Total lipid and water content agree with findings for midwater fishes with gas-filled swimbladders²⁶.

I compared the metabolic energy requirements of *C. armatus* with the stored body energy in the form of neutral lipid and glycogen. Assuming a respiratory quotient of 0.75 because of the greater percentage of stored neutral lipid (93%) to glycogen (7%) in these rattails and the high lipid content of the preferred food of a fish of this size^{27,28}, and a mean respiration rate of 2.5 ml O₂ per h (Table 1), I obtained a daily energy requirement of 0.28 kcalories. The protein metabolism component (excretion rate) was ignored because it constitutes only 3–6% of the energy requirement calculated from oxygen consumption. The mean neutral lipid (5.11 g×9.30 kcalories per g=47.55 kcalories) and glycogen (1.13 g×4.19 kcalories per g=4.73) content of the three rattails (Table 2), when added and then divided by the daily energy requirement, shows that this stored energy could meet these animals' energetic requirements for 186 d. This estimate certainly is consistent with the hypothesis that little food is available in the deep sea.

My results and the ensuing calculations suggest that *Coryphaenoides armatus* is adapted to the deep sea by having a low metabolic rate and sufficient stored energy to exist for extended periods without eating. From the evidence accumulated so far on activity rates in the deep sea, these adaptations seem essential.

Table 2 Chemical composition of three individuals of *C. armatus*

Component (%)	<i>Coryphaenoides armatus</i>		
	1	2	3
Water content	78.2	88.2	81.0
Lipid			
total	1.3	0.9	1.1
neutral	0.9	0.6	0.7
other	0.4	0.3	0.4
Glycogen	0.2	0.2	0.1

All components are given in percentage of total wet weight. Gut contents were removed before proximate analyses. Data are from refs 18–20, 29 and 30.

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Interest of mice in conspecific male odours is influenced by degree of kinship

SMELL affects many aspects of the social and sexual behaviour of rodents^{1,2}. For example, fighting in pairs of male mice is increased if one of the participants is made to smell unfamiliar by rubbing it with the urine of a strange male³. It has also been suggested that olfactory cues play an important part in mate choice⁴. In both rats⁵ and mice⁶, females which have been reared by artificially scented mothers have, when adult, been found to prefer males treated with this same scent to normal individuals. By contrast, Mainardi⁷ has found that female mice, reared in the presence of their fathers, later preferred to mate with males of a different strain. The difference between these two results may stem from the degree of strangeness involved, females preferring males which smell slightly unfamiliar rather than very familiar or totally strange. The results of the experiments using artificial odours suggest that early learning provides the yardstick by which unfamiliarity is judged^{5,6}. Such a system may have an important role in assortative mating,

enabling females given a choice to mate with those males by whom they are likely to conceive the best-adapted offspring. On this hypothesis females might be expected to prefer a small degree of unfamiliarity as an adaptation which minimises the chance of inbreeding. They might also be expected to reject males which smell very strange. At an extreme, this would avoid interspecific hybridisation, but it may also be disadvantageous within a species for very dissimilar individuals to mate as this would be likely to lead to the break-up of co-adapted gene complexes and thus the production of offspring of lower fitness^{8,9}. We report here our examination of these predictions. Our data suggest that the olfactory preferences of female mice are consistent with this hypothesis.

Eighteen female mice (10 Porton homozygous normal and 8 Steel homozygous normal), reared in the presence of their fathers, were isolated at weaning and tested when 10 weeks of age. Both the test animals and those used to provide olfactory stimuli were kept in identical individual cages in the same rack, and supplied throughout with food, water and sawdust litter. The animal to be tested was placed in the middle of an arena (48×54 cm) in the floor of which were four holes 7 cm in diameter arranged in a square and with a glass pot sunk in each. One of these (pot c) contained fresh sawdust as a control; the other three contained sawdust which had been used as litter on the floors of the cages of three different individual males for 3-7 d immediately before the test. These males had the following relationship to the animal being tested: a brother (pot b), a member of the same strain which was not a brother (pot ss) and a member of a different strain, this being the other of the two strains used (pot ds). Each animal was tested with sawdust from the same three individuals for 5 min on four consecutive days to minimise any effects which the oestrous cycle might have on odour preferences. The tests were carried out under dim red light early in the dark phase of the animals' light-dark cycle. The total time for which the head of the mouse protruded over the side of each pot was recorded in 0.1-s units using a keyboard connected to four counters. The apparatus was washed out after every trial and the positions of pots randomised between trials. The same procedure was adopted using 10 males (six Porton and four Steel) to determine whether the sexes reacted differently, and the results of both experiments were subjected to a three-way analysis of variance (pots×days×subjects). Figure 1 shows the mean responses of males and females to the four different pots.

The female subjects showed no significant difference between the four days of testing ($F=0.63$, df 3/51), nor was there a significant interaction between days and pots ($F=1.38$, df 9/153). There was, however, a highly significant difference between the pots ($F=14.72$, df 3/51, $P<0.001$). That this was not solely attributable to lack of interest in the pot containing fresh sawdust is shown by the fact that the difference remained significant when the analysis was repeated excluding this pot ($F=4.88$, df 2/34, $P<0.05$). The females showed greatest interest in the pot containing sawdust from the cage floor of the male which belonged to the same strain but was not their brother (Fig. 1).

The male group was smaller as fewer males had brothers from which cage-floor sawdust could be obtained. The results were also less clear. There was a significant difference between the days of testing ($F=5.90$, df 3/27, $P<0.01$), which was not simply due to habituation, as day 1 provided the second lowest mean score. There was also a significant interaction between pots and days ($F=3.60$, df 9/81, $P<0.01$), pot ss being the most investigated on day 1, pot ds on day 2 and pot b on day 3. This result is not easy to interpret, but avoidance of the strangest smell on day 1 may stem from initial nervousness, whereas attention to the most familiar one on day 3 may be because the others have, by then, been thoroughly investigated. Despite these complications, the difference between pots was also significant, though less so than the other effects ($F=3.68$, df 3/27, $P<0.05$); it also ceased to be significant when the control pot (pot c) was excluded from the calculations ($F=$

3.40, df 2/18). Thus, although the data show a tendency for greater investigation of the smells of less closely related individuals, and this was consistent between strains (Fig. 1), the evidence is not conclusive.

The trend which the male mice show towards interest in the pots containing the smells of non-relatives may simply result from their familiarity with their own smell and with that of sawdust. The more genetically different from them another male is, the more his smell is likely to diverge from these familiar odours and the more investigation it may therefore demand. By contrast, the female mice tested showed a clearer but less simple pattern of preference, with most interest being taken in the smell of males which were from the same strain as themselves but were not their brothers. This bears out the original prediction and suggests that the olfactory preferences of female mice may provide a mechanism whereby they select males whose genes are most likely to combine with their own to give the fittest offspring. Mainardi's finding⁷ that females preferred to mate with males of a different strain may be because the strains which he used were more genetically similar than those used here. Testing the possibility that females discriminate between males in the way suggested here will require mate choice experiments in which the genetic relationship between the participants is known precisely and varied systematically. There is already evidence that the mating preferences of male mice are influenced by genetic differences in the *H-2* complex of their partners¹⁰; the female deserves closer

study as her greater investment in the offspring suggests that she has more to lose by a poor selection of mate and that she should therefore be more discriminating.

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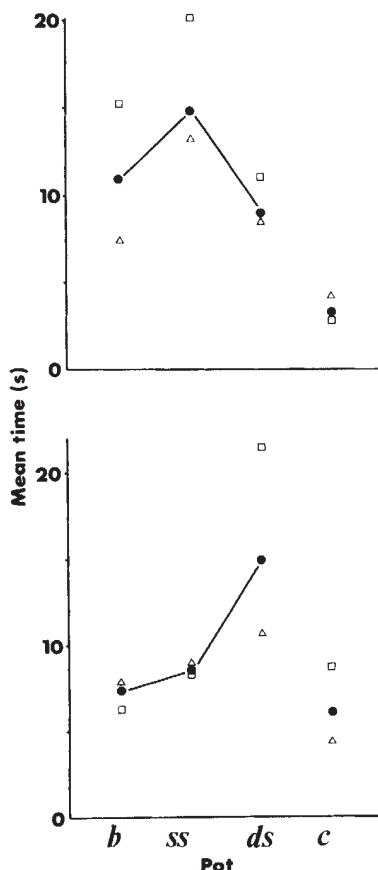
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Fig. 1 Mean investigation time per 5-min test shown by female (top) and male (bottom) mice to four different pots. Pots contained: sawdust from the floor of the cage of a brother (pot *b*), from that of a male of the same strain who was not a brother (pot *ss*), from that of a male of the other strain tested (pot *ds*) and fresh sawdust (pot *c*). Investigation times plotted are: ●, mean for all mice; △, mean for Porton homozygous normal mice; □, mean for Steel homozygous normal mice.



An athymic nude mutation in the rat

IN 1953 an athymic hairless mutation was observed in a colony of outbred hooded rats maintained at the Rowett Research Institute. A breeding colony of mutant animals was maintained with difficulty until the early 1960s, when it died out. However, the recessive mutant gene (to which the symbol *mu* for Rowett nude has been assigned) was apparently maintained at low gene frequency within the random bred colony. Homozygous mutants were recovered in 1975, and a breeding colony was again established, first in conventional, and later in germ-free conditions, where good breeding performance and survival were obtained. In February 1977 a breeding nucleus of these rats was established at the MRC Laboratory Animals Centre in conventional, germ-free and specific pathogen-free conditions. The observations and data reported here were obtained with small numbers of animals bred in clean, conventional conditions, and suggest the potential usefulness of nude rats in biomedical research.

A homozygous nude rat is shown in Fig. 1. Vibrissae are present, but are bent, a feature that may be used to recognise homozygotes soon after birth (as in nude mice). Some short hairs are often found on the head, and to a lesser extent on the rest of the body, though there is considerable individual variability. From about 2 to 6 weeks of age (with considerable variation) the skin often seems to be covered with brownish scaly flakes, but the rats later become smooth and hairless. At later ages some animals may grow a substantial covering of hair (though this is always much less than a normal coat), but this hair is soon unevenly lost again. Internally, no gross abnormalities have been observed except that the thymus is replaced by brown fat tissue, though as in the nude mouse, there is a small and highly abnormal thymus rudiment. Detailed histology has not yet been investigated in any organs, but preliminary observations of lymph nodes and Peyer's patches show depletion of lymphocytes in T-lymphocyte-dependent regions.

Growth of nude rats is reduced to about 60–80% of their heterozygous littermates, although in the colony described here there has been no evidence of the runting syndrome frequently seen in conventional nude mice. Lifespan of nude rats has not been determined. Preweaning mortality of nude rats is higher than that of their heterozygous littermates, but in the absence of any specific pathological infection postweaning losses have been negligible. Two out of four homozygous nude males born in November 1976 are still alive, and are therefore over 17



Fig. 1 A homozygous nude rat.

months old. Small numbers of homozygous nude rats have been placed in the same room as conventional (non-SPF) rats from about 4–8 weeks of age, and have survived well for up to 5 months, but the numbers are too small to show anything other than the fact that these animals can survive well in such conditions. An outbreak of Tyzzer's disease was associated with the death of several homozygous nude rats, but the conventional colony was discontinued before any serious losses were observed, in order to protect other colonies on site.

Homozygous nude rats of both sexes are fertile. So far, only two females have been allowed to breed, and both have produced several litters, but have only reared small numbers of young. The usual breeding method has been to mate homozygous (*rru/rru*) males with heterozygous (*rru/+*) females, the system commonly used in the production of nude mice¹. However, it might be possible to develop a line of nude rats in which homozygous females breed reasonably efficiently.

Allografts from inbred rats having the *H-1^f*, *H-1^c* and *H-1^m* haplotypes at the major histocompatibility complex were given to a single homozygous female nude rat, and were not rejected after 3 months. The Rowett Hooded outbred stock, in which the mutation is maintained, was later found to be segregating for the *H-1^f* and *H-1^c* haplotypes (J. C. Howard, personal communication), although the genotype of the recipient is not known. Xenografts of tail skin from a gerbil (*Meriones unguiculatus*) were placed on the tail of a single homozygous nude male. These grafts healed in well, and have not been rejected after a period of 4 months, although they are now somewhat reduced in size and have lost their hair.

Studies by Ford and Smith² of two nude rats show that output of thoracic duct lymphocytes (TDL) 24 h after cannulation was reduced to 15–25% of control values. Normal TDL were injected intravenously and the same amount were recovered from the thoracic duct of nude recipients as from normal recipients. Nude rat TDL recirculated slowly but equally in nude and normal recipients. Autoradiographs of the spleens and lymph nodes of nude or normal recipients of either nude or normal rat TDL showed that the localisation of these cells was as would have been expected if the nude rat TDL were entirely B lymphocytes.

Several studies in progress indicate that the nude rat, like the nude mouse, has depressed or absent T-cell function. In particular, the immune responses to the T-cell mitogens concanavalin A and phytohaemagglutinin are substantially reduced or nonexistent in homozygous nude rats, whereas there is a normal response to *S. aureus*, a B-cell mitogen (C. Brooks, personal communication); nude rats are killed within 10–13 d by an infection of the larvae of the tapeworm *Taenia crassiceps* (J. Chernin, personal communication) that does not normally kill immunologically competent rats; the output of oocysts after an initial challenge with the coccidian parasite *Eimeria nieschulzi* is significantly increased in homozygous nude rats compared with heterozygotes or normal rats; and in

contrast to normal rats there is no reduction in oocyst output after the second and third challenge (E. Rose, personal communication).

Small-scale attempts to grow tumours in homozygous nude rats have had mixed success. A plasma-cell tumour specific to BALB/c mice, and a human colon tumour both grew for a short time in two nude rats and then regressed, but the reason for this regression has not been studied. However, the TLX5 lymphoma which is a rapidly growing tumour specific to CBA/Ca mice grew rapidly in 4/6 nude rats, and in one case attained a weight of 17 g 10 d after the subcutaneous injection of 10^5 ascites cells in the inguinal region. However, in the CBA mouse this tumour spreads throughout the body, whereas in the nude rat the tumour largely remained localised. When samples of the tumour were subsequently reinjected into CBA mice the growth remained localised. Histological studies of this tumour grown in nude rats have not yet been carried out, but further studies involving this tumour are planned.

Preliminary studies in the nude rats of the chemically induced HSN_{TC} fibrosarcoma, which normally only grows in the Chester Beatty hooded inbred strain of rats, have established that it grows well in the homozygotes but not in heterozygous nude rats (which are maintained on a genetically variable background). There was a substantial reduction in mononuclear phagocyte infiltration which only accounted for about 5–7% of the cells in the tumour (S. Eccles, personal communication) compared with at least 30% in the Chester Beatty rats; also, there was no measurable antibody response (C. Dean and S. Hobbs, personal communication).

Further research will be necessary to determine whether there are any T cells in the nude rat, and to what extent there is still a cell-mediated immune response. However, the ability of the nude rat to accept xenografts of skin and tumours, the highly abnormal response to parasitic infections and the substantial reduction in response to T-cell mitogens clearly suggest that the nude rat is in many ways similar to the nude mouse first described in detail in 1966 (ref. 3) and found to be athymic in 1968 (ref. 4). The nude mouse is now the most widely used pathological mutant in biomedical research, including cancer research, immunology and the study of infectious disease⁵. The nude rat should become widely used in these three areas, and should be particularly useful in studies involving surgery (for which the nude mouse is often too small), and in any of these areas in which the rat is the preferred species. It may also prove to be more robust than the nude mouse, which is extremely sensitive to common murine viruses such as murine hepatitis and Sendai viruses, which are apparently not a serious problem in rats. Breeding nuclei of these rats are being made available as quickly as possible.

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Involvement of histocompatibility antigens in embryonic cell recognition events

OBSERVATIONS of developmental events such as the sorting out of cells during organogenesis suggest that highly specific mechanisms exist for cells to migrate apart or aggregate together. Several assays of cell-to-cell binding using cells from developing tissues have provided probable *in vitro* analogues of these recognition mechanisms. When cells from various organs of the embryo are dissociated, re-aggregation occurs in a tissue-specific manner with, for example, liver cells binding preferentially to other liver cells and brain cells binding preferentially to other brain cells. A number of groups using different assay systems have agreed with this general finding¹⁻⁴. Furthermore, our own work with chick embryonic neural retina cells has shown that proteolytic enzyme treatment of these cells removes structures which are necessary for specific cell-cell adhesion to occur⁵. The relevant 'recognition' moieties on the cell surface may be glycoproteins⁶⁻⁸. We have investigated cell-cell adhesion using embryonic tissues from two strains of mice, C57B1/10 and C3H, which differ from one another at the *H-2* locus as well as at a number of minor *H*-loci.

Much of the work on specificity of cell adhesion has used chick embryo cells; however, tissue-specific cell adhesion (*in vitro*) has been observed in a variety of other species including the mouse⁹. The mouse is of special interest because of the vast quantity of genetic information available on cell-surface molecules in this species. In particular, the highly polymorphic histocompatibility-2 (*H-2*) locus codes for cell-surface glycoproteins which have been implicated in 'self-recognition' by the immune system¹⁰.

A number of other histocompatibility loci of more limited polymorphism have been characterised immunologically; as yet, however, biological function for most of these antigens can not be assigned. Where investigated, *H*-locus antigens are found on cell surfaces and are expressed in different amounts in different tissues. It is possible that some or all of these loci code for products which are involved in embryonic cell recognition. If this were the case, then one might predict that cells which differ at the relevant *H*-locus would adhere to one another less well in an *in vitro* assay than would homologous cells.

Our approach has utilised a cell collection assay¹¹ in which single cells are collected to the surface of cell aggregates. A number of investigations have shown this assay to be very

sensitive to apparent specificity differences between embryonic tissues¹¹⁻¹⁴. We have modified the assay to reflect relative specificity differences between cells of two types. That is, the assay permits us to quantify specificity so that numbers can be assigned to recognition differences. These recognition index (RI) numbers then can be used not only to show whether a difference exists, but also to show the relative magnitude of a specificity difference with respect to other tissue comparisons.

Brain and liver tissues were dissected from 15-16 d C3H/HeJ, and C57 B1/10 SnJ mouse embryos. The tissues were minced mechanically and were dissociated with trypsin (0.03% in calcium-magnesium-free tyrodes solutions)⁵; the cell suspensions were aggregated overnight in 25 ml flasks in Eagles BME+5% fetal calf serum. Conditions were adjusted so that more than 95% of the cells were viable, and a starting suspension of 6×10^6 cells ml⁻¹ in 3 ml produced several hundred aggregates with diameters of between 80-150 μ m. Labelled cell suspensions were prepared as follows: 1-mm tissue cubes were incubated for 16 h in either ¹⁴C-leucine (4 μ Ci ml⁻¹) or ³H-leucine (10 μ Ci ml⁻¹) in leucine-free Eagles BME.

The labelled tissues were dissociated and the resulting cell suspensions were incubated in sparse culture at fast (115 r.p.m.) rotation speeds for 4 h. This preincubation of cells permitted repair from trypsin effects at the surface of the labelled cells but the rotation speed inhibited cell interactions⁵. The labelled cells were collected and added to the flasks containing aggregates. Identical aliquots of cells were added to two flasks of each pair to be compared for specificity. For example, if brain and liver were being compared, a flask containing brain cell aggregates and a flask containing liver cell aggregates each received identical aliquots of ³H-labelled brain cells and ¹⁴C-labelled liver cells. The two flasks were then incubated at 37 °C for 1 h at 70 r.p.m. At the end of this time the aggregates were separated from any single cells remaining in suspension using a 73 μ m Nitex membrane filter (Kressilk, New York) especially constructed for this purpose⁶. The aggregates, containing collected ³H-labelled and ¹⁴C-labelled cells were then precipitated on glassfibre filters and counted by scintillation methods in a PPO-POPOP-toluene mixture¹¹.

The data were converted to a RI by the following calculations. First, d.p.m. values were calculated for ³H and ¹⁴C, and then background subtractions were made. The ratio of homo-typic/heterotypic cell counts collected was determined for each pair of flasks. The two ratios [for example, (3H d.p.m. (brain)/¹⁴C d.p.m. (liver)) collected to brain aggregates, (¹⁴C d.p.m. (liver)/³H d.p.m. (brain)) collected to liver aggregates] were multiplied to yield an RI; all units (d.p.m., cell number, and so on) cancel out, leaving a number that reflects a relationship

Table 1 Different mouse histocompatibility types

Cell type 1	Cell type 2	Aggregate type 1	Aggregate type 2	Collection to aggregate type 1 Cell 1/cell 2	Collection to aggregate type 2 Cell 1/cell 2	RI†
C3H liver	C3H liver	C3H liver	C3H liver	1.048 ± 0.08*	0.979 ± 0.1*	1.013 ± 0.10
C3H liver	C57B1/10 liver	C3H liver	C57B1/10 liver	1.675 ± 0.063	1.743 ± 0.026	0.960 ± 0.038
C57B1/10 liver	C3H liver	C57B1/10 liver	C3H liver	1.349 ± 0.12	0.912 ± 0.16	1.479 ± 0.29
C57B1/10 brain	C57B1/10 liver	C57B1/10 brain	C57B1/10 liver	13.801 ± 0.47	4.187 ± 0.28	3.29 ± 0.24
C57B1/10 liver	C57B1/10 brain	C57B1/10 liver	C57B1/10 brain	2.362 ± 0.43	0.491 ± 0.02	4.81 ± 0.80
C3H brain	C57B1/10 brain	C3H brain	C57B1/10 brain	1.61 ± 0.07	1.66 ± 0.1	0.970 ± 0.072
C57B1/10 brain	C3H brain	C57B1/10 brain	C3H brain	2.16 ± 0.06	2.15 ± 0.41	1.004 ± 0.19

* Each number represents the mean ± s.e.m. of three determinations per sample. Cell type 1 was labelled with ³H and cell type 2 was labelled with ¹⁴C. In each case reciprocal labelling was carried out and results of two comparisons of each combination are shown.

† RI, recognition index = [(cell type 1/cell type 2) to aggregate 1] × [(cell type 2/cell type 1) to aggregate 2].

between two cell types. In practice, if the two tissues being compared are identical then the RI should be 1. Table 1 shows that this is indeed the case. It follows that the greater the difference in recognition between homotypic and heterotypic cells, the larger the RI. In the case of brain and liver the RI is relatively high (Table 1), indicating that there is very little cross-recognition between these two tissues. In other examples, we have shown high indices between cells of different species of sea urchin embryos and between different chick embryonic tissues (unpublished). A lower RI is seen between different neural tissues (unpublished) and between neural tissues of the chick and the mouse⁸. Thus, using this method we have been able to compare, in pairs, a variety of embryonic tissues to determine the extent to which cells of these tissues recognise each other.

Tissues from C3H mice and C57B1/10 mice are known to express allelic differences at the major histocompatibility locus (*H-2*) as well as five out of eight minor *H* loci¹⁵. We felt that if histocompatibility antigens were involved in recognition, a comparison between tissues that express a large number of different alleles should give us a difference in RI. Using this experimental approach we have examined embryonic liver and brain. Table 1 shows no significant difference in RI in C3H brain compared with C57B1/10 brain and a small difference in RI in C3H liver compared with C57B1/10 liver. This difference may be real but the RI was very close to 1 in four different assays (1 ± 0.2). Therefore if there is an adhesive specificity difference between liver cells of different histocompatibility backgrounds, it is too low to demonstrate convincingly with a collection assay. These data show therefore, that even with large differences in histocompatibility background, homotypic cell recognition properties are not affected significantly.

Even if histocompatibility antigens were to participate in embryonic cell recognition phenomena, the multiplicity of histocompatibility alleles is hard to explain. Within an individual, cells express two alleles of a given gene locus. If histo-

compatibility alleles were to be used for cellular recognition events, it could be argued that the genetically variable portion of the molecules must be unimportant for recognition events. Otherwise an already complicated morphogenetic series of events would be further complicated by individual differences that would have to be recognised.

A further argument against the involvement of the *H*-locus antigens in cell adhesive interactions is the fact that *H-2* specificity is relatively insensitive to trypsin whereas adhesive events are highly sensitive to trypsin digestion (Fig. 1). When we compared *H-2* antigens on 15-d embryonic liver cells after dissociating the tissue either mechanically or with trypsin, we observed no difference between the two cell suspensions (Fig. 1). *H-2* antigens were clearly demonstrable on 15-d embryonic liver cells (Fig. 1); on 15-d brain cells *H-2* antigens either were not present or were present at levels below the limits of detection by the ⁵¹Cr assay (data not shown).

The experiments presented above do not rule out possible involvement of histocompatibility antigens in embryonic recognition events. These experiments do show, however, that large genetic variability in histocompatibility expression does not affect *in vitro* recognition events as measured by a highly quantitative assay.

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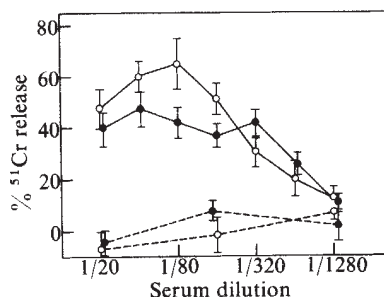


Fig. 1 Expression of *H-2* on fetal liver cells. A single cell suspension of 15-d C3H/HeJ liver was prepared and allowed to recover from trypsinisation for 4 h^{14,5}. Other cells were treated with trypsin and used immediately. Both trypsinised and non-trypsinised cells (10^7 each) were labelled with 450 μ Ci $\text{Na}_2^{51}\text{CrO}_4$ in 1 ml minimal essential Eagles medium with 10% fetal calf serum (MEM10) for 60 min at 37 °C and then washed three times at 4 °C. Antibody-mediated lysis of these cells was measured in Microtest II plates as follows: 2×10^5 cells in 20 μ l MEM10 were incubated in triplicate with 20 μ l diluted C3H.SW/SnJ anti-C3H/HeJ serum or with normal C3H.SW/SnJ serum at 37 °C for 45 min. Rabbit complement (10 μ l 1:2 dilution absorbed with C3H/HeJ spleen cells at 1:5 packed cells:complement for 1 h on ice) was added and the mixture incubated for 45 min at 37 °C. Cold PBS (50 μ l) was then added; the plates were centrifuged at 600g for 10 min, and 50- μ l aliquots were counted in a Beckman Gamma-4000 counter. Results are expressed as percentage ⁵¹Cr release = (c.p.m. released by serum - c.p.m. released by complement alone)/(c.p.m. released by 1 M HCl - c.p.m. released by complement alone) $\pm$ s.e.m. ●, Non-trypsinised liver; ○, trypsinised liver. Solid line, anti-H-2 serum; dashed line, normal mouse serum.

Isolation of antibodies to HCG/LH from human sera

ANTIBODIES to gonadotrophin have previously been detected only in the sera of a few patients, mainly with endocrinological disorders¹⁻⁴ or receiving hormone therapy, and antibodies reacting with trophoblast have been described in association with pregnancy⁵⁻⁸. We report here the presence of antibodies to human chorionic gonadotrophin (HCG) and luteinising hormone (LH) in normal human sera. These were found initially in pooled postpartum sera but sera from males, non-pregnant females, children and umbilical cord also contained antibodies which were predominantly IgG. HCG is a secretory product of trophoblast and forms an integral part of the trophoblast membrane⁹⁻¹². Its distinction from human pituitary LH lies principally in the β chain where HCG has an additional C-terminal 30 amino acids¹³.

Antibodies to HCG/LH were isolated by affinity chromatography. Isolated antibodies were tested by reaction with ¹²⁵I-HCG and by precipitation of the resulting complex with anti-human IgG. Results of assays of binding of ¹²⁵I-HCG to anti-

bodies isolated from normal sera are presented in Table 1. Isolated antibodies had low avidity and binding was rarely detected at antibody concentrations of $<10 \mu\text{g ml}^{-1}$.

An attempt was made to determine quantitatively the anti-HCG/LH content of sera using a solid-phase radioimmunoassay with HCG-coated tubes and ^{125}I -anti-human IgG (Fig. 1). A wide range of values was obtained with normal adult sera and sera from some children and from some patients with various types of cancer had no anti-HCG/LH antibodies.

We were unable to demonstrate antibodies to HCG/LH in any serum without use of a solid support for HCG as described above. No such antibodies were detected when serum was incubated with ^{125}I -labelled HCG followed by Sephadex G-200 chromatography or electrophoresis on cellulose acetate. This could be attributable to the low avidity of the antibodies or to the action of inhibitors, possibly serum gonadotrophins. Interestingly, a low molecular weight fraction (MW $\sim 10,000$) from normal serum could inhibit binding of ^{125}I -HCG to isolated anti-HCG/LH.

The specificity of binding of ^{125}I -HCG to isolated anti-HCG/LH was studied by inhibition of binding with gonadotrophins, their subunits and with thyroid stimulating hormone (TSH). Figure 2 shows that the isolated antibody is specific for intact HCG and LH and that less inhibition was obtained with α and β subunits of HCG. FSH and TSH were not inhibitory at the concentrations shown but were inhibitory in large excess.

We tried to exclude the possibility that the isolated antibodies were directed against blood group substances since

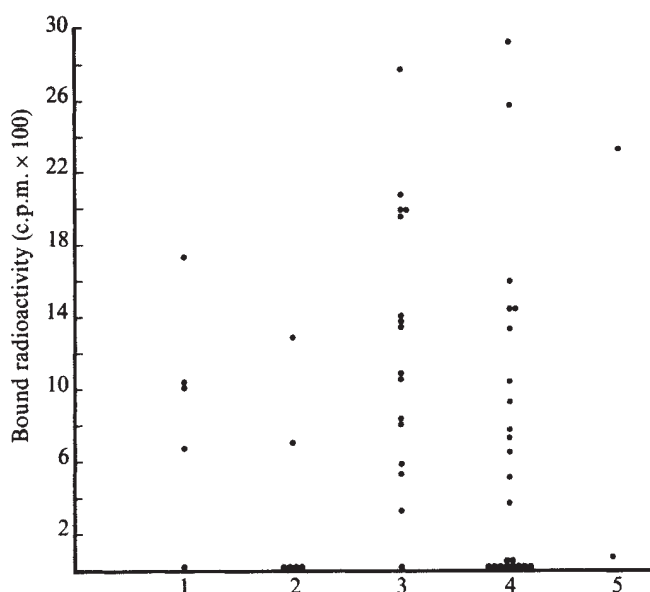


Fig. 1 Solid phase assay of anti-HCG/LH: 50 μl aliquots of 1 mg ml^{-1} aqueous HCG solution (Pregnyl, $2,000 \text{ IU mg}^{-1}$) were added to round-bottomed polystyrene tubes and the tubes were left at 37°C to evaporate to dryness. Tubes were then treated at 4°C with 200 μl cold 10% aqueous formaldehyde for 10 min emptied and washed with 0.1 M Tris-0.5 M NaCl buffer, pH 7.85 (Tris buffer). Tubes were left filled with Tris buffer at 4°C until required. Serum under test was diluted 1/200 with Tris buffer containing 0.5% bovine serum albumin (dilution buffer) and 100 μl were added to each coated tube. Tubes were incubated at 37°C overnight, and then washed 10 times with Tris buffer containing 0.5% Tween 20. 100 μl ^{125}I -anti-human IgG ($\sim 170,000 \text{ c.p.m.}$) was added to each tube for further 5 h. Tubes were washed again 10 times with Tris buffer-0.5% Tween 20, emptied and counted. Controls for nonspecific binding were treated in the same manner and consisted of: empty tubes; HCG-coated tubes where serum was replaced with dilution buffer; tubes coated with α -fetoprotein, also at 50 μg per tube; and tubes where serum was replaced with rabbit anti-HCG serum, diluted 1/200. The number of counts per tube was corrected for nonspecific binding by subtracting the counts found in empty tube controls. Each point in the histogram is a mean of duplicate determinations. 1, Sera from pregnant women; 2, sera from children aged 4 to 10 yr; 3, normal adult sera; 4, sera from patients with various carcinomas before and after treatment; 5, pooled sera from 20 lactating women.

Table 1 Binding of ^{125}I -HCG to the isolated anti-HCG/LH

Column	Serum	Protein desorbed (μg) per 1 ml serum	Protein concentration tested ($\mu\text{g ml}^{-1}$)	% Binding
1	Normal female	ND*	60	50
1	Normal male	ND	60	41
1	Lactating pool	ND	60	65
2	T.A., normal female	130	130	52
2	F.B., normal female	170	170	24
2	M.W., normal female	110	110	26
2	A.K., normal male	124	124	22
2	G.G., normal male	138	138	18
2	Cord serum	96	96	10

Antibodies were isolated by affinity chromatography on column 1 or column 2. Column 1 was packed with 150 mg HCG ($1,800 \text{ IU mg}^{-1}$) bound to Sepharose and equilibrated with 0.1 M phosphate/0.5 M NaCl buffer, pH 7.35 (equilibration buffer, EB). Serum samples (10–50 ml single serum or serum pools) were precipitated with ammonium sulphate to 50% saturation, the precipitate was dissolved in saline, dialysed against saline to remove ammonium sulphate, and then dialysed against EB. Samples were centrifuged at 23,000 r.p.m. to remove denatured protein and placed on the column. Unadsorbed protein was eluted with EB until A_{280} fell to base level. Retained protein was eluted using 2.5 M ammonium thiocyanate in EB with pH adjusted to 7.35. Desorbed protein was dialysed immediately against saline and the volume of solution was reduced by ultrafiltration through a PM-10 membrane. Column 2 was packed with 25 mg highly purified HCG ($5,000 \text{ IU mg}^{-1}$) bound to Sepharose. Equilibration buffer and desorbing buffer were as for column 1. In column 2 serum samples (5 ml) were applied directly without prior treatment with ammonium sulphate. The retained protein peak was dialysed against saline and volume reduced to 5 ml by ultrafiltration. Protein concentration was measured as A_{280} with a γ -globulin standard. The conditions for assay of ^{125}I -HCG binding were: 25 μl anti-HCG solution, 25 μl 5% anti-human IgG, 50 μl ^{125}I -HCG ($\sim 100,000 \text{ c.p.m.}$) and 100 μl 50 mM phosphate buffer containing 0.1% bovine serum albumin, incubated overnight at 37°C . The sample was filtered and the number of counts found in the precipitate was corrected for nonspecific binding. Binding is expressed as counts bound as percentage of total counts added.

* ND—Not determined.

HCG has been reported to have blood group A activity in its α subunit¹⁴. No decrease in the binding of ^{125}I -HCG occurred after prior absorption of isolated anti-HCG/LH with blood group A erythrocytes, nor after absorption of T antigen with neuraminidase-treated blood group O erythrocytes¹⁵.

The properties of isolated anti-HCG/LH were studied. Although there were some IgM and IgA class antibodies the predominant IgG class was evident by its binding to Protein A-Sepharose and by its behaviour in SDS-polyacrylamide gel electrophoresis. In gel diffusion the rabbit antiserum to isolated anti-HCG/LH gave a single line against a 1/40 dilution of normal human serum. This precipitin line was continuous with the line given by human IgG standard at $250 \mu\text{g ml}^{-1}$. Complement binding was tested by the method of Bradstreet and Taylor¹⁶ and anti-HCG/LH from all three normal sera tested were positive.

Isolated anti-HCG/LH did not react in gel diffusion against HCG at various concentration ratios, and no precipitin lines were obtained in gels containing 4% polyethylene glycol.

The physiological significance of the presence of anti-HCG/LH antibodies in most human sera is not clear. They may be involved in the regulation of activity of gonadotrophins—by retarding their loss from the blood, for instance.

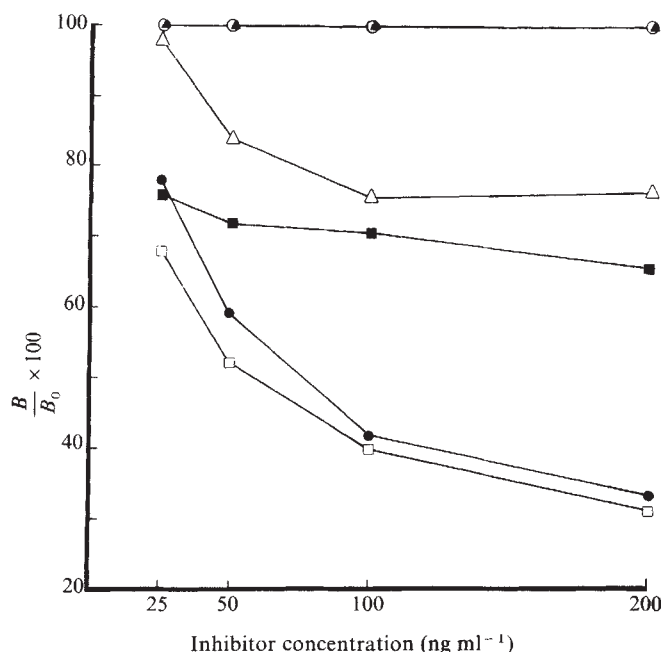


Fig. 2 Inhibition curves for anti-HCG/LH from normal adult serum. ○, TSH; ▲, FSH; △, α subunit of HCG; ■, β subunit of HCG; ●, HCG; □, LH. B_0 = binding in absence of inhibitor, B = binding in presence of inhibitor.

Low-affinity antibodies to hormones have been reported in patients undergoing hormone therapy and have been found to protect the hormone from rapid destruction^{17,18}. Low-affinity antibodies in relatively high concentrations would be no obstacle to hormone action, as they would not compete with high-affinity gonadotrophin receptors in target organs.

The formation of these anti-hormone antibodies seems to provide evidence of autoimmunity to self-proteins circulating in very low concentrations, as postulated by Weigle¹⁹ and Allison²⁰.

In contrast to the anti-HCG antibodies resulting from immunisation procedures, natural anti-HCG/LH antibodies do not seem to interfere with conception. Early secretion of HCG at and after implantation may contribute towards neutralisation of any harmful effect that such antibodies might have on early embryos. The significance of the abnormally low levels of anti-HCG/LH antibody in some cancer patients is not yet known. Evidence that HCG secretion in pregnancy may interfere with immune responses has proved controversial²¹ but it is interesting that antibodies to human placental lactogen have been found in postpartum and normal female sera²². A further possibility is that anti-HCG/LH antibodies may be provoked by microorganisms which share some antigenic sites with HCG. Antibodies to mycobacteria and various unrelated bacteria are present in sera from humans with no history of exposure to these microorganisms²³. The sharing of antigens between microorganisms and certain mammalian cells^{24,25} or cancer cells²⁶ has been reported. Especially pertinent to our study are the reports^{27,28} of HCG-producing bacteria isolated from tissues of patients bearing malignant neoplasms.

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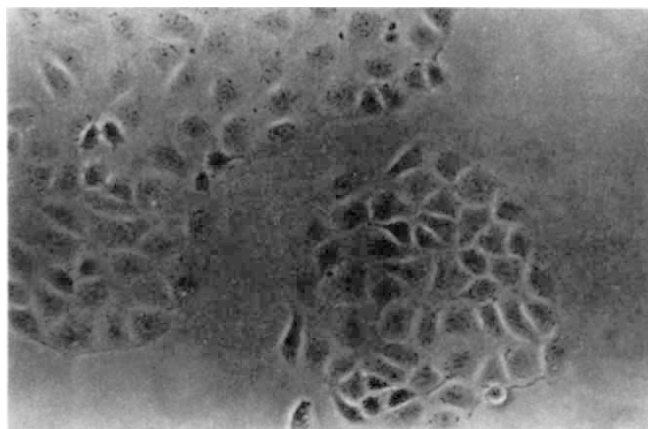
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Malignant transformation of a mouse epithelial cell line with polycyclic aromatic hydrocarbons

MALIGNANT transformation of cells in culture can serve as a means for studying cellular and molecular mechanisms of chemical carcinogenesis and as a method for screening for environmental carcinogens (see refs 1, 2 for reviews). Although most of these systems involve fibroblast cultures from mouse, rat and hamster embryos³⁻⁵, there are few reports of the malignant transformation of epithelial cell cultures except with liver cells^{6,7}. However, there are some inherent difficulties in working with epithelial cells derived from liver, a highly specialised organ with a multitude of enzyme activities. Many of these enzymes tend to decrease in activity in long-term culture, and may not be equivalent biochemically to their state *in vivo*. Thus, biochemical studies of carcinogenesis are difficult with such cells, and there are no reports of a normal hepatocyte line that can be transformed in culture and produce a hepatoma on inoculation into the syngeneic host. There are some reports of malignant epithelial cell lines that originated from treating primary cultures obtained from urinary bladder, trachea, kidney, prostate and submandibular gland with different chemical

Fig. 1 Nonconfluent growth of MC-3T3 cells showing compact areas of growth of polygonal cells with granular cytoplasm and prominent nucleoli. ($\times 75$).



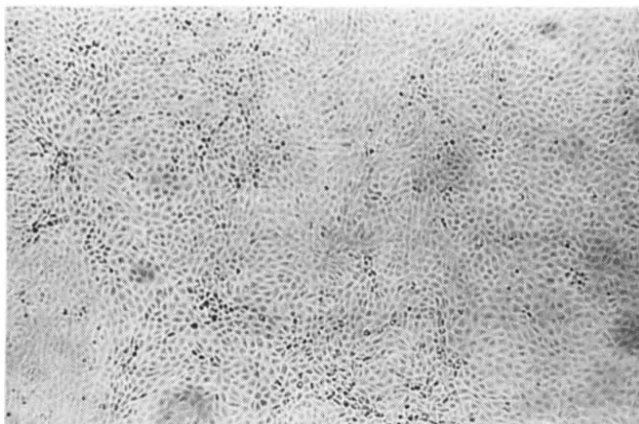
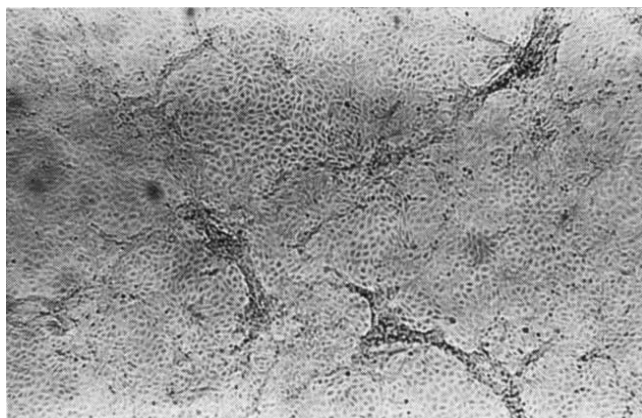


Fig. 2 Confluent monolayer control, acetone-treated, MC-3T3 cells with marked contact inhibition of growth at 6th week of continuous culture. ($\times 30$).

carcinogens⁸⁻¹³. In such cases, however, there are no normal counterparts available for comparison of biological properties. So far as we know, no line of epithelial cells apart from liver cells has transformed with chemical carcinogen and given rise to carcinomas when inoculated in the appropriate host. We now describe an epithelial cell line from wild mouse embryo, which can be transformed with polycyclic aromatic hydrocarbons. These transformed cells produce poorly differentiated carcinomas when inoculated into nude mice.

The new cell line (MC 3T3) was developed by U. Rapp from the embryos of a wild mouse, *Mus musculus castaneus*, by using 3T3 schedule¹⁴ and has been characterised and cloned several times by us. The cells are polygonal with a large nucleus and prominent nucleolus, and the cytoplasm shows some granularity. They grow in compact colonies (Fig. 1) before they reach confluence, when they assume a 'cobblestone' pattern (Fig. 2). The transformation experiments were done by seeding 2,000 cells into 60-mm plastic dishes (Falcon) in minimum essential medium (MEM, Gibco) supplemented with 10% fetal calf serum (FCS, Gibco). The next day the medium was removed and the cells fed with medium containing various polycyclic aromatic hydrocarbons at different concentrations. The concentration of the carcinogen used for transformation was selected after toxicity experiments. 3-Methylcholanthrene (MCA) was used at $5 \mu\text{g ml}^{-1}$, which gave a relative plating efficiency (RPE) of 50%, and $2.5 \mu\text{g ml}^{-1}$, RPE 66%; benzo[a]pyrene (BP) $2.5 \mu\text{g ml}^{-1}$, RPE 61%; and 7,12-dimethylbenz[a]anthracene (DMBA) $1 \mu\text{g ml}^{-1}$, RPE 33%. The cells were treated with carcinogens for 24 h, then washed

Fig. 3 Areas of piled up foci on the surface monolayer at 6th week of culture of MC-3T3 cells treated with $1 \mu\text{g ml}^{-1}$ DMBA for 24 h 1 day after seeding. ($\times 30$).



and fed with normal medium (MEM + 10% FCS). Control cells were treated with 0.5% acetone, the solvent for the carcinogen, in medium for 24 h and then fed with the normal medium. The cultures were then fed twice weekly until they reached confluence, and then once a week with normal medium. After 5 weeks scattered piled-up areas of more refractile cells of various sizes appeared in all carcinogen-treated dishes. These areas were small, 0.2–1 mm in diameter, and were multiple (Fig. 3). It was not possible to isolate them separately for culture. After 6 weeks the treated cultures were trypsinised and passed with 100,000 cells in 75 ml T-flasks. Six flasks were prepared for each series after the cells were pooled. Three more passages were carried out in the same way at intervals of 10–12 d. With each passage, the piled-up areas increased both in size and in number in all the carcinogen-treated dishes. The control cultures showed no such areas. The cells from the fourth passage were collected by trypsinisation (0.1%) and were injected into nude mice (USC:NIH(S)). From the first experiment with $5 \mu\text{g ml}^{-1}$ MCA, six mice were injected subcutaneously with 10^6 cells per mouse and six mice were injected with 10^6 acetone-treated cells per mouse. Within 3 weeks all the mice injected with MCA-treated cells developed progressively growing tumours at the site of injection, and none of the mice injected with acetone-treated cells developed any tumours in 6 months. From the second experiment, four

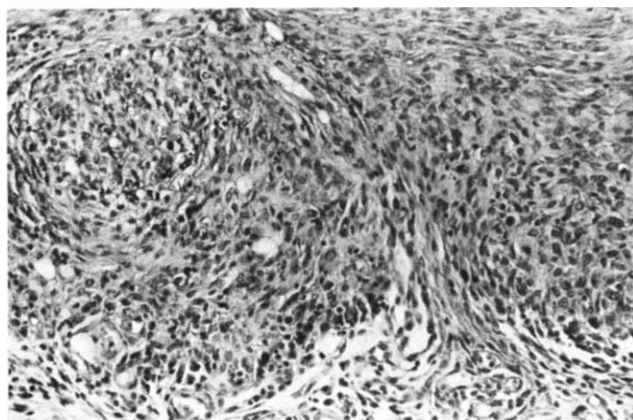


Fig. 4 Histological picture of the tumour developed in nude mice after injection of DMBA treated cells. It shows poorly differentiated carcinoma of uncertain origin. ($\times 48$).

mice were injected with $1.5\text{--}2 \times 10^6$ cells per mouse from each series of MCA, BP, DMBA and acetone-treated cells. Within 3–4 weeks all of the mice injected with MCA, BP and DMBA treated cells developed progressively growing local tumours at the site of injection. After 14 weeks, 1 mouse out of 4 injected with acetone-treated cells showed evidence of a tumour at the site of injection. When the tumours developed from carcinogen-treated cells grew to 1–2 cm in diameter, they were excised for histological examination. The sections showed poorly differentiated carcinomas (Fig. 4). The origin of the cells is not certain as they were derived from a culture of whole embryos. In these experiments we could not calculate a transformation frequency, because the transformed foci were small and multiple in the dishes. Work on transformation with other carcinogens, determination of transformation frequency and studies of the properties of the normal and transformed cells are in progress.

It is estimated that 90% of human cancers are epithelial in origin, so it is important to have an epithelial cell line that can be transformed by carcinogens, so that cellular and molecular mechanisms of carcinogenesis can be studied and compared with those of the fibroblast systems. We have now such an epithelial cell system.

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Haemin promotes rapid neurite outgrowth in cultured mouse neuroblastoma cells

MOUSE neuroblastoma cells grown in suspension culture are round and anaplastic, but when grown in culture attached to a surface and in the presence of various inducing agents such as dibutyl cyclic AMP¹, bromodeoxyuridine², prostaglandin E₁ (ref. 3), cytosine arabinoside⁴, papaverine⁵, dimethyl sulphoxide (DMSO)^{6,7}, hexamethylene bisacetamide^{6,7}, or serum-free medium^{8,9}, the cells will extend neurites and assume a morphology resembling that of the mature neurone. The mechanism of neurite outgrowth is unknown. Neurite outgrowth develops slowly in the presence of the defined agonists, usually requiring one to several days to attain the optimum outgrowth. Thus, the possible importance of cell-cycle associated events and cell division can complicate the interpretation of experimental results. And, with serum deprivation, nonspecific alterations may result from the removal of numerous factors in the complex mixture comprising serum. Therefore, the availability of a rapidly acting agonist effective in the presence of the normal complement of serum would be a useful tool. We report here that haemin can rapidly and reversibly induce neurite outgrowth in cultured mouse neuroblastoma cells and suggest that haemin may have a wider role in cells than previously thought.

The cultured mouse neuroblastoma cell is a useful model for the study of morphological differentiation. The cell line used here, clone Neuro 2a(ATCC CCL131), was initially established by Klebe and Ruddle¹⁰. The cells are electrically excitable¹¹, and contain adrenergic and cholinergic enzymes^{10,12,13}. N2aH is our subline which has been adapted to growth in the presence of HEPES buffer. The conditions of cell culture and seeding in preparation for experimentation are described in detail elsewhere¹⁴. Plastic microwell plates containing monolayers of N2aH cells in the log phase of growth were washed twice with 0.5 ml Hanks' salts solution immediately before experimentation. The percentage of cells bearing neurites was determined by counting several random fields under phase contrast microscopy. Only cells with processes greater than or equal to the diameter of the perikaryon (about 40 µm) were scored as

positive; cells with multiple processes were scored only once. Between 120 and 160 cells were counted in each culture.

The spontaneous level of neurite outgrowth was approximately 2%, whereas when 10⁻⁴ M haemin was present neurite outgrowth increased to about 22% in 3 h (Fig. 1a). This level of neurite outgrowth was maintained for at least 6 h. After 1 day in the presence of haemin, neurite outgrowth remained elevated over that of control cultures. The morphology of haemin-responsive cells appears the same as that of cells demonstrating spontaneous neurite outgrowth in untreated cultures. The absolute level of both induced and spontaneous neurite outgrowth can vary somewhat between experiments. The concentration range within which haemin is active is quite narrow—neurite outgrowth was stimulated at concentrations above 10⁻⁵ M with an optimum at 10⁻⁴ M (Fig. 1b). Concentrations greater than the optimum resulted in a reduced proportion of neurite-bearing cells.

The induced outgrowth is completely reversible (Table 1). Cultures incubated in the presence of 10⁻⁴ M haemin for 3 h contained about 25% neurite-bearing cells as compared to 2% for unexposed cultures. The outgrowth returned to baseline values within 3 h in cultures from which haemin had been washed out.

Fig. 1 (Left) Time and concentration-dependent effect of haemin on neurite outgrowth in cultured mouse neuroblastoma cells. Haemin (Sigma) was dissolved in 1M KOH then adjusted to pH 7.4-7.8 with HCl. Stock solutions (20 mM) were divided into aliquots and stored at -20 °C. In *a*, cells were incubated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (DMEM) in the presence (●) or absence (○) of 10⁻⁴ M haemin. The proportion of neurite-bearing cells in the cultures was determined at various times. In *b*, the cells were incubated for 3 h in the DMEM medium in the absence (○) and in the presence (●) of various haemin concentrations. The proportion of neurite-bearing cells in the cultures was scored. Values are means ± s.e.m. (*n* = 4).

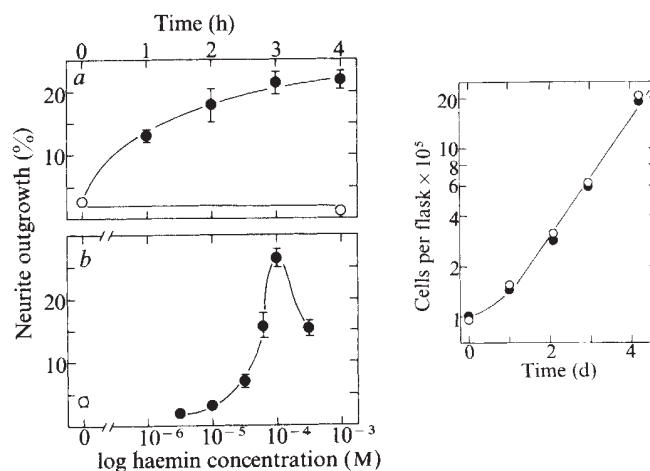


Fig. 2 (Right) Effect of prior exposure to haemin on the growth rate of neuroblastoma cells in culture. Cells were incubated for 3 h in DMEM medium in the presence (●) or absence (○) of 10⁻⁴ M haemin. The cells were then detached by incubation for 15 min at 37 °C in Hanks' salts solution with 1 mM EDTA and collected by centrifugation at 600g for 5 min. After resuspension in fresh growth medium (RPMI 1640 with 10% fetal calf serum), 10⁵ cells were seeded onto Falcon plastic 25-cm² tissue culture flasks. Each day for 4 days thereafter, the incubation medium and the cells which had been detached by incubation for 2 min at 37 °C in Hanks' salts solution containing 1 mM EDTA and 0.05% trypsin, were combined and collected by centrifugation. The cells were resuspended in 1 ml of growth medium and an aliquot counted in a haemocytometer. The values are the means (*n* = 4). Standard errors are not shown as they were smaller than the ranges encompassed by the symbols.

Table 1 Retraction of neurite outgrowth after washout of haemin in cultured mouse neuroblastoma cells

Treatment		Neurite outgrowth (%)*	
0 to 3 h	3 to 6 h	At 3 h	At 6 h
-Haemin	-Haemin	2.0 ± 0.9	2.0 ± 0.9
+Haemin (10 ⁻⁴ M)	+Haemin (10 ⁻⁴ M)	24.5 ± 0.7	19.9 ± 4.1
	-Haemin		3.2 ± 2.3

Cells were incubated in DMEMS medium in the presence or absence of 10⁻⁴ M haemin for 3 h and the proportion of neurite-bearing cells was scored. All cultures were then washed twice with Hanks' salts solution, and incubated for an additional 3 h in the same conditions as before, except that one group previously exposed to haemin was incubated in its absence. Neurite outgrowth was scored again after 6 h. * Values are means ± s.e.m. (n = 4).

The optimum response obtained at 10⁻⁴ M haemin is not related to a cytotoxic effect. There was no difference in the ability to exclude Trypan blue dye between cells exposed to haemin for 24 h and control cells. The growth rate of cells previously incubated for 3 h in the presence or absence of 10⁻⁴ M haemin is shown in Fig. 2. Between 1 and 4 days, the doubling time of the cultures was ~1 day; no significant difference was apparent between the growth rates of the two cultures. Whether the diminution of the response at high haemin concentrations is due to a cytotoxic effect is not clear.

Several compounds related to haemin either structurally or as intermediates in haemin metabolism were studied in order to establish the specificity of the response (Table 2a). In haemin ferric iron is chelated in a planar complex by a tetrapyrrole ring system. Pyrrole and porphobilinogen were incapable of promoting neurite extension. Biliverdin, a degradation intermediate of haemin, was marginally active (9%). Protoporphyrin, the iron-deficient precursor of haemin, was tested separately (data not shown) and could promote neurite outgrowth. The haemin structure is not uniquely effective since chlorophyllin, at 10⁻⁴ M could promote 25% and at 2 × 10⁻⁴ M about 31% neurite outgrowth. Chlorophyllin is a complex which does not contain iron, and differs further from haemin by the presence of a fused cyclopentanone ring in the plane of the tetrapyrrole ring system, and different substituents on the

Table 2 Ability of compounds structurally or metabolically related to haemin to promote neurite outgrowth and effect of KCN, NaN₃ and NaF on response to haemin in cultured mouse neuroblastoma cells

Treatment	Neurite outgrowth (%)*
<i>a, Control</i>	
Haemin (10 ⁻⁴ M)	4.3 ± 1.1
Biliverdin (10 ⁻⁴ M)	42.1 ± 1.9
Porphobilinogen (10 ⁻⁴ M)	9.0 ± 1.4
Chlorophyllin (5 × 10 ⁻⁵ M)	6.3 ± 1.1
Chlorophyllin (10 ⁻⁴ M)	15.9 ± 2.1
Chlorophyllin (2 × 10 ⁻⁴ M)	24.6 ± 1.7
Pyrrole (10 ⁻⁴ M)	30.9 ± 1.1
	4.1 ± 0.4
<i>b, Control</i>	
Haemin (10 ⁻⁴ M)	2.7 ± 0.2
KCN (5 × 10 ⁻⁴ M)	43.0 ± 2.2
NaN ₃ (5 × 10 ⁻⁴ M)	10.7 ± 1.1
NaF (5 × 10 ⁻⁴ M)	11.3 ± 1.3
Haemin (10 ⁻⁴ M) plus KCN (5 × 10 ⁻⁴ M)	10.9 ± 0.8
Haemin (10 ⁻⁴ M) plus NaN ₃ (5 × 10 ⁻⁴ M)	40.1 ± 1.8
Haemin (10 ⁻⁴ M) plus NaF (5 × 10 ⁻⁴ M)	39.1 ± 2.4
	33.4 ± 1.4

Cells were incubated for 3 h in DMEMS medium with additions as indicated. Control cultures in (a) received 0.02% ethanol, which served as the solvent for compounds used in that experiment. The proportion of neurite-bearing cells in the cultures was determined.

* Values are means ± s.e.m. (n = 4).

various pyrrole moieties. Chlorophyllin may have toxic effects, however, as the cells showed an abnormal, grainy appearance.

To further test the importance of the central iron atom in haemin, compounds which could form ferric complexes were examined (Table 2b). The azide and cyanide complexes seem to be as active as haemin itself, suggesting that the iron atom does not play a major part in promoting neurite outgrowth. Considering the concentrations of inhibitors used¹⁵, it seems probable that the normal rate of mitochondrial respiratory chain function, or rapid activity of certain metalloenzymes such as peroxidase or catalase, is not required.

These studies show that haemin can rapidly and reversibly induce neurite outgrowth in cultured mouse neuroblastoma cells in a manner unrelated to cytotoxicity. Variations in the level of neurite outgrowth attained may depend on the presence of inhibitory factors associated with the lipid component of serum¹⁶, or on fluctuations in the metabolic state of the cell. We infer that the tetrapyrrole ring structure is minimally required for the haemin effect; the presence of the central iron atom seems to be unimportant. Induction of neurite outgrowth is not restricted to the mouse neuroblastoma cell, as embryonic chick dorsal root ganglia cells are also responsive to haemin (data not shown).

The mechanism by which haemin induces neurite outgrowth is not clear. DMSO can induce aspects of differentiation in both neuroblastoma and murine erythroleukaemia cells, so one possibility is that haemin acts through membrane stabilisation, a mechanism suggested for DMSO in erythroleukaemia cells¹⁷. Haemin has not been found to cause membrane stabilisation in any system, but the possibility remains open. However, considering the following observations, we favour an alternate explanation. Globin and haem synthesis are coordinated in erythroid cells, and haemin at 10⁻⁴ M can promote globin synthesis in intact reticulocytes^{18,19}. It also stimulates total protein synthesis in reticulocytes and in Krebs II ascites tumour cells²⁰⁻²². Studies with cell-free extracts have shown that stimulation of protein synthesis by haemin occurs either as a result of direct inhibition of a cyclic AMP-independent protein kinase which inactivates the eukaryotic initiation factor, eIF-2, or, occurs indirectly through non-competitive binding to the regulatory subunit of a cyclic AMP-dependent protein kinase^{23,24}. Furthermore, haemin inhibits protein kinase activity associated with chromatin preparations from rat liver²⁵. The observation that haemin can regulate the activity of protein kinases, and the large body of experimental evidence indicating a relationship between phosphorylative modification of proteins and expression of differentiated states^{26,27}, suggests that in the neuroblastoma cell haemin may act through protein kinases to promote the assembly or synthesis of structural proteins, or both. Haemin may thus have a wider role in the cell than is currently appreciated. The induction of globin mRNA accumulation in erythroleukaemia cells^{28,29} and of neurite outgrowth in neuroblastoma cells by haemin are similar in that in both systems haemin is the most rapidly acting agonist yet observed and in that maximum induction occurs at similar concentrations. Hence, the expression of the differentiated state may result, at least in part, from mechanisms common to both cell types.

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Structural and functional restoration by collateral sprouting of hippocampal 5-HT axons

DAMAGED 5-HT (5-hydroxytryptamine) axons in the central nervous system have been observed to undergo vigorous sprouting^{1,2}. Regeneration of these axons to their original termination sites has been seen several months after intraventricular administration of selective 5-HT neurotoxic drugs; and in the spinal cord this regrowth has been correlated with a return of normal 5-HT receptor function as measured by a hindlimb stretch reflex³. These findings raise two questions. First, is the pattern of re-innervation after injury dependent on some regenerative property of the damaged axons themselves, or is it primarily controlled by the denervated terminal field? And second, can re-innervation take place not only by regeneration of the original axons but also by collateral sprouting of chemically identical but anatomically separate undamaged fibres; and, if so, will function be restored? In the work reported here we have taken advantage of the fact that the hippocampus receives two separate inputs of 5-HT fibres from the median raphe nucleus, and we have shown that the unilateral removal of one input induces re-innervation of the unoccupied sites by the other. This collateral sprouting occurring in a single hippocampus is accompanied by disappearance of an asymmetrical behavioural response, suggesting that not only structural but functional recovery has occurred. Our results, therefore, suggest that the 5-HT terminal field plays a major part in the reorganisation of this system after injury to the brain.

The 5-HT projections to the hippocampus were selected as investigations have shown that the 5-HT axons in this area are derived from two different subpopulations in the midbrain median raphe nucleus, and enter the hippocampus by two discrete routes: a supracallosal one which travels above the corpus callosum with the cingulum bundle, and an infracallosal projection travelling below the corpus callosum with the fornix-fimbria system^{4,5}.

We were able to destroy 5-HT fibres in either bundle by direct microinjection of 5,7-dihydroxytryptamine (5,7-DHT) into them, which in the conditions used here, removed 5-HT but left noradrenaline fibres unaffected (as determined by measurements of the uptake tritiated monoamines into hippocampal slices). As ³H-proline injected into the raphe nuclei is

transported to the terminal sites of these 5-HT-producing neurones, we were able to assess anatomically the effects of the lesions by autoradiography. To test the functional effects of the lesions, we made use of the suggestion that the hippocampus might function as a novelty detector⁶ and investigated whether unilateral hippocampal dysfunction might influence the direction of turning shown by rats in an unfamiliar situation.

Ninety-eight adult CFHB rats (230–250 g) pretreated with the catecholamine uptake blocker desmethylamphetamine hydrochloride (DMI, 10 mg per kg body weight), were injected unilaterally in the cingulum bundle at a lateral angle of 15° to avoid the superior sagittal sinus (coordinates from bregma: AP-1.0 mm, V-2.4 mm, L-1.2 mm). 5,7-DHT (5 µg free base in 0.4 µl of Ringers containing 0.2 mg ml⁻¹ ascorbic acid) was passed through a glass pipette (tip diameter 50–80 µm) at a rate of 50 nl min⁻¹. This injection technique was shown by uptake measurements of ³H-5-HT and ³H-noradrenaline to spare catecholamine axons (Table 1); ultrastructural and electrophysiological studies revealed that non-monoaminergic fibres and the surrounding neuropil were similarly spared (E.C.A., Buchan, Williams, McNaughton, and Gray, in preparation). At various times after cingulum bundle injections (3, 6, 10, 15, 21, 28, 56 and 90 days) ³H-proline (10–25 µCi per 0.2–0.3 µl saline) was injected into the median raphe nucleus at a rate of 20 nl min⁻¹ using a 30° posterior angle to avoid passing near the cingulate cortex and the confluence of the sinuses. These rats were perfused 24 h later with 10% formalin in phosphate buffer (pH 7.2) and the brains processed for autoradiography. The hippocampus on the uninjected side of each rat served as its own control.

After ³H-proline injection into the midbrain median raphe nucleus, silver grains were observed in the normal hippocampus both in Ammon's horn and in the dentate gyrus, the highest density being found in the polymorphic layer of the latter region. In rats examined between 1 and 14 days after 5,7-DHT microinjection into the cingulum bundle the grains in the dorsal-most part of the hippocampus were reduced on the injected side, while in more ventral parts the grain distribution remained normal (Fig. 1, (1c)). This was most evident in the polymorphic region, and therefore we selected this region to compare lesioned and control hippocampi. In other rats, injections were made into the fornix-fimbria, at 15° lateral approach, and in these animals the grains in this dorsal-most polymorphic region remained, despite reduced 5-HT uptake in the dorsal hippocampus (Table 1). This observation illustrates the differential innervation of the hippocampus by these two 5-HT projections in normal animals.

However, rats, left for 28–90 days after 5,7-DHT microinjections into one cingulum bundle, showed a similar distribution of grains in the dentate gyrus on both lesioned and

Table 1 Dorsal hippocampal uptake of ³H-5-HT and ³H-NA

	5-HT	NA
Sham (n = 6)	446 ± 53 (100%)	382 ± 47 (100%)
Cingulum (n = 6)	310 ± 33 (70%)	389 ± 26 (102%)
Fimbria (n = 6)	250 ± 37 (59%)	369 ± 50 (96%)

Uptake of ³H-5-HT or ³H-NA by hippocampal slices 10 days after injections 5 µg 5,7-DHT into either the cingulum bundle or the midline fimbria-fornix of adult female CFHB Wistar rats (250 g) pretreated 1 h previously with DMI. The hippocampi were rapidly removed from a fresh brain and the dorsal half cut into six slices (1 mm thick). These were incubated in Krebs buffer (pH 7.2) with either 5 × 10⁻⁸ M ³H-5-HT or ³H-NA for 12 min at 37 °C. Controls were left at 0 °C after the addition of the radioactive substrates. The samples were weighed, dissolved in 0.8 ml Soluene and counted in 10 ml toluene with Triton. The values are given as c.p.m. ± s.e. per mg wet tissue.

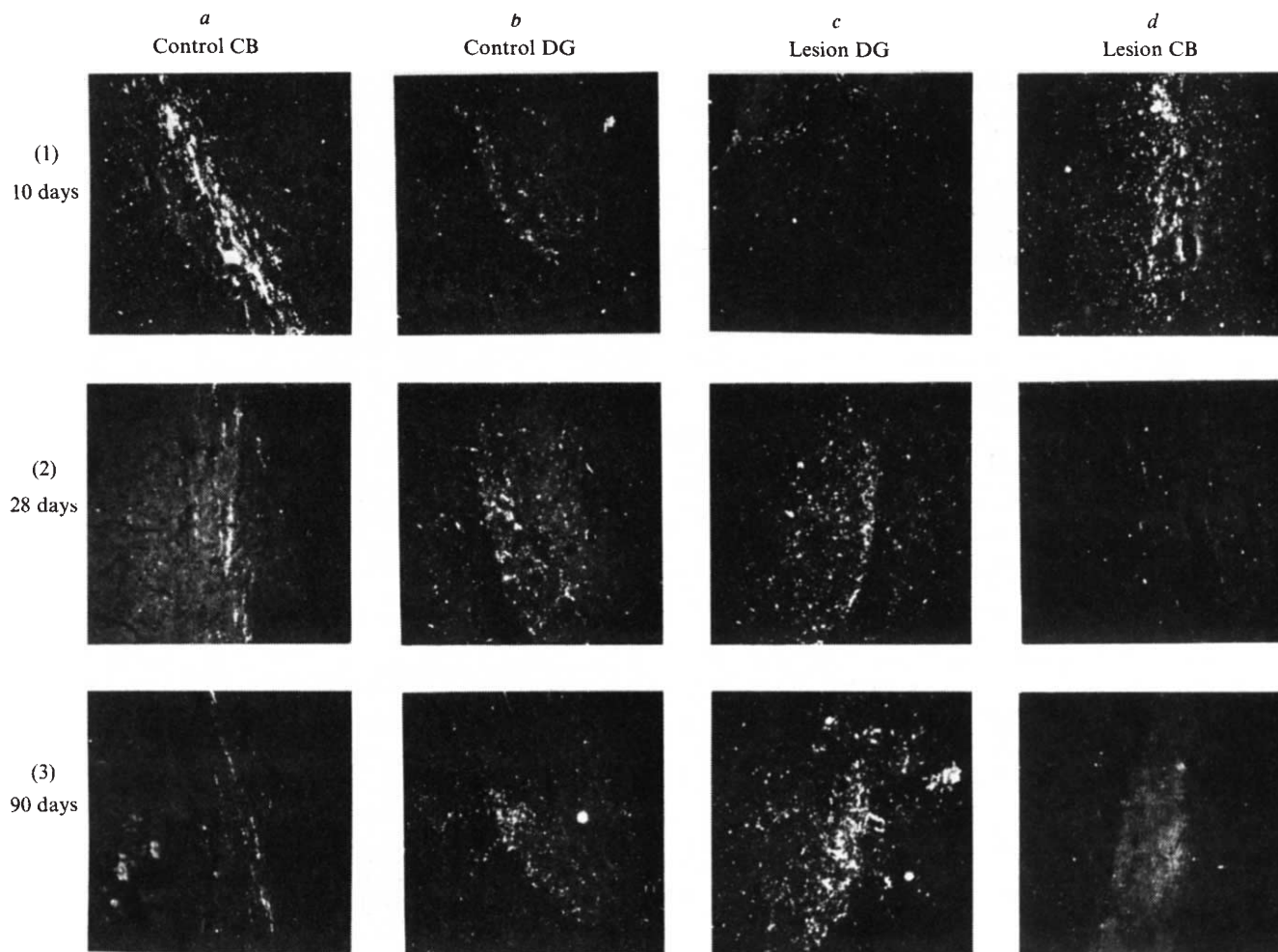


Fig. 1 Horizontal sections of the dentate gyrus in adult female rats (250 g) at various times (10, 28 and 90 days) after unilateral cingulum bundle microinjections of 5,7-DHT ($5 \mu\text{g}$ in $0.5 \mu\text{l}$). The brains were sectioned at $5 \mu\text{m}$ and Ilford K2 emulsion (1:1 dilution with 2% glycerol in distilled water) used; the slides exposed at 5°C for 5 weeks before development (freshly prepared Phen-X, undiluted, 2 min). Silver grains are found in the control uninjected cingulum bundle (1a) and in the polymorphic layer of the dentate gyrus (1b). At 10 days after 5,7-DHT injections into the right cingulum bundle, labelling both in it (1c) and the dentate gyrus (1d) was markedly reduced. After 28–90 days the uninjected cingulum bundle (2a, 3a) and the dentate gyrus (2b, 3b) are again labelled, but the 5,7-DHT injected cingulum bundle remained substantially depleted at 28 days (2d) and at 90 days (3d). However at both these times grains were found in the polymorphic region of the injected side (2c, 3c). ($\times 25$, dark field illumination.)

control side (Fig. 2, (2)b, c and (3)b, c). Inspection of the cingulum bundle distal to the injection site in these rats revealed no reappearance of grains, indicating that regeneration of these damaged axons did not occur (Fig. 1, (2) and (3)d).

In order to correlate these structural findings with equivalent functional ones, we had to choose a behaviour which could reveal a unilateral hippocampal dysfunction. We considered that turning in an unfamiliar environment might be such a behaviour as the hippocampus has been implicated in novelty detection⁶.

Rats were placed in a circular bowl (30 cm in diameter) and observed from an adjoining room for 20 min, both before and 30 min after the injection of L-5-hydroxytryptophan (5-HTP, 50 mg per kg, intraperitoneally). By analogy with findings using L-DOPA⁷, this treatment will result in a specific stimulation of 5-HT receptors⁸. Animals were tested at between 1200 and 1800 hours. Three days after neurotoxic drug microinjections, the rats turned to the left (contralateral) side before 5-HTP and to the right (ipsilateral) side after 5-HTP (Fig. 2). Sham-injected rats showed no differences in turning in the two directions before or after 5-HTP. At 14–28 days after microinjection there were no differences in turning before, but a

marked contralateral turning after 5-HTP. These observations suggest that 5-HT receptors had become supersensitive to 5-HT on the lesioned side by day 14, which is similar to findings on 5-HT receptors in the spinal cord³ and on acetylcholine receptors in muscle fibres⁹. At longer survival times (42–56 d) after lesion the rats no longer displayed any consistent direction of turning before or after 5-HTP administration (Fig. 2). The disappearance of this asymmetrical behavioural response to 5-HTP occurred at the same time as the reappearance of silver grains in the dentate gyrus (Fig. 3). We confirmed our idea that collateral sprouting from the infracallosal 5-HT axons was responsible for the disappearance of the behavioural effects in these longer-term animals by making a second neurotoxin injection into the midline of the fornix-fimbria. (coordinates from bregma; AP–1.1 mm, L–0.9 mm, V–4.5 mm) (Fig. 3). Such an injection, by itself, had no effect on turning behaviour; however, when carried out on rats in which unilateral cingulum bundle fibres had been damaged 42 d previously, strong contralateral turning again reappeared after 5-HTP had been given (Fig. 2).

We consider that the change in turning response is not due to generalised motor dysfunction because the animals' total activity remained unimpaired after unilateral cingulum bundle

injection and the asymmetrical turning behaviour continued to habituate rapidly over the 20 min test period (75% occurring in the first 4 min). As other areas (the cingulate cortex) supplied by these cingulum 5-HT fibres would not be affected by damage to the fornix-fimbria, it seems likely that our behavioural results were due to the anatomical changes seen in the hippocampus. This suggestion is strengthened by the observed correlation between behaviour and hippocampal 5-HT depletion. At 14 days after cingulum bundle microinjections there was a correlation between decreased 5-HT uptake in the dorsal hippocampus and the change in unilateral turning after 5-HTP in a group of 6 rats ($r.s. = 0.89$; $P < 0.05$; one-tailed). Furthermore a similar correlation was observed in the animals which received the double lesion procedure shown in Fig. 2 ($r.s. = 1.0$; $P < 0.01$; one-tailed).

The ability of the infracallosal 5-HT axons to assume the function of the supracallosal 5-HT axons in restoring symmetry in turning behaviour suggests that the function performed by these fibres is not unique to either projection. This situation is similar to that found by Langley for autonomic nerve fibres in the peripheral nervous system¹⁰. He concluded "that the function of any autonomic nerve fibre depends, not so much upon its inherent properties as upon the nerve cells with which it has an opportunity of becoming connected in the process of development". Thus, the function of the serotonergic nerve fibres in the central nervous system may depend on the inherent properties of the neural systems with which they connect. This could explain why manipulation of whole brain 5-HT influences such a large number of behaviours. For

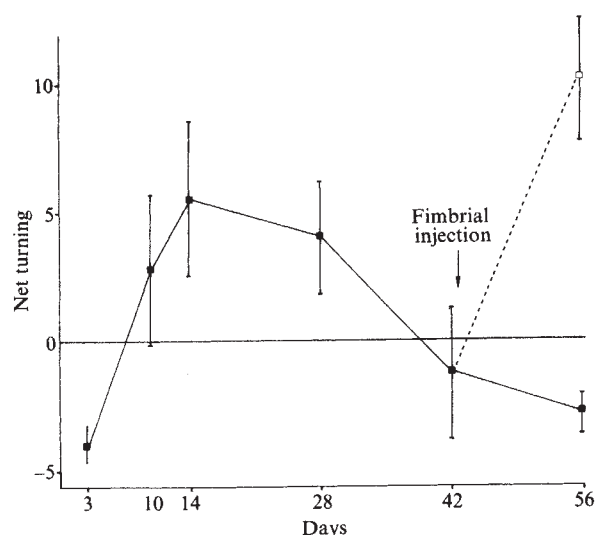


Fig. 2 The net turning (TN) at various times after unilateral injection of 5,7-DHT into the right cingulum bundle (supracallosal 5-HT fibres) is expressed as the difference in 180° turns before (od) and after 50 mg per kg 5-hydroxytryptophan (d) ($TN = (R-L)_{od} - (R-L)_d$). At 3 days ($n = 6$) the rats showed increased left (contralateral) turning before and right (ipsilateral) turning after 5-HTP. However at 10 ($n = 6$), 14 ($n = 23$), and 28 ($n = 17$) days after 5,7-DHT the situation is reversed with rats displaying no difference in turning before, but a markedly increased left (contralateral) turning after 5-HTP injection. The net turning at 42 ($n = 16$) and 56 ($n = 5$) days after cingulum bundle injections of 5,7-DHT demonstrates that the rats are no longer showing any significant asymmetrical turning behaviour. However if a midline injection of 5,7-DHT is made into the fimbria (infracallosal 5-HT fibres) in rats ($n = 5$) 42 days after the unilateral cingulum bundle injection, then the net turning (dotted line) is similar to that observed 14 d after the cingulum bundle injection alone (STCB) but significantly different from a comparable group of long-term cingulum bundle (LTCB) rats (56 days). Analysis of variance—two-way interaction (day/treatment), d.f. = 3, 180; $F = 6.555$; $P < 0.001$; two-tailed.

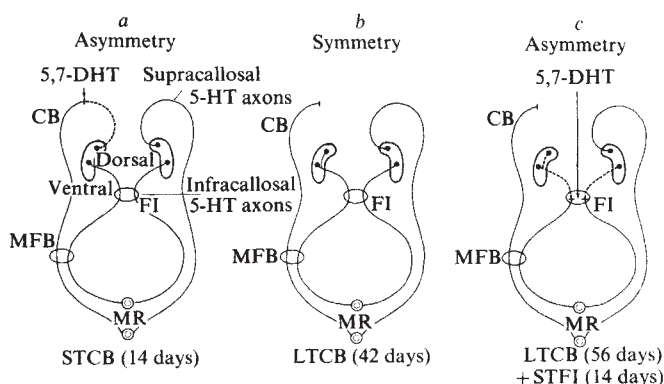


Fig. 3 A schematic representation of the 5-HT innervation of the hippocampus by supracallosal fibres travelling in the cingulum bundle (CB) and by infracallosal fibres travelling in the fimbria (FI). These fibres originate from different neurones in the median raphe nucleus and ascend to the forebrain in the medial forebrain bundle (MFB). *a*, The degenerating 5-HT axons (dashed line) after unilateral short-term cingulum bundle (STCB) microinjection of 5,7-DHT, and the resultant anatomical asymmetry produced. *b*, The collateral sprouting of the infracallosal 5-HT axons into the dorsal hippocampus which restores symmetry (after long-term cingulum bundle (LTCB) injection of 5,7-DHT). *c*, The degenerating 5-HT axons (dashed line) produced by a midline microinjection of 5,7-DHT into the fimbria (STFI) of LTCB rats and the resultant asymmetry produced. The midline microinjection into the fimbria of normal rats (not shown) produced no such asymmetry.

instance, 5-HT has been shown to be involved in the regulation of sleep, temperature, pain, aggression, locomotor activity, self-stimulation, sexual behaviour, avoidance-learning and water consumption¹¹. Thus it is possible that any one of these behaviours may be selectively modulated by the localised manipulations of 5-HT within the brain structure subserving that particular behaviour.

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Serially homologous neurones as concomitants of functional specialisation

MOST higher invertebrates and all vertebrates are metamerically segmented and their evolution has depended on the progressive functional specialisation of serially repeated, originally similar segments. In structurally similar body segments, serially homologous neurones have been shown to have remained largely unchanged¹⁻⁴. Is this also the case for serially homologous neurones in segments which show important morphological and functional differences? We report here that we have identified motoneurones innervating muscles in the first two pairs of legs of the locust *Schistocerca gregaria*, Forskal (= *S. americana*, Dirsch). These legs are similar and represent the primitive unspecialised form, functioning especially in posture and walking. The hind legs are greatly enlarged and specialised for jumping, and motoneurones innervating muscles in these legs have been identified⁵. In walking, the legs in a segment move alternatively, whereas in jumping, both hind legs act almost synchronously⁶. We have found that serially homologous motoneurones have evolved new functional properties for jumping while retaining their primeval morphologies.

Motoneurones innervating the flexor tibiae (FITi) and extensor tibiae (ETi) muscles in each of the three pairs of legs

Fig. 1 *a*, FITi and ETi motoneurone cell bodies are shown at their average location in the three thoracic ganglia. The six FITis shown are the anterior fast (AFFITi), anterior intermediate (AIFITi), anterior slow (ASFITi), posterior fast (PFFITi), posterior intermediate (PIFITi), and posterior slow (PSFITi). FETi and SETi motoneurone cell bodies are seen to have switched positions in the metathoracic ganglion relative to their location in the pro- and mesothoracic ganglia. Metathoracic FITi and ETi cell body locations are from ref. 5. Calibration, 100 μ m. *b*, Camera lucida drawings of PFFITi shows their very similar morphologies in each of the three thoracic ganglia. Calibration, 200 μ m.

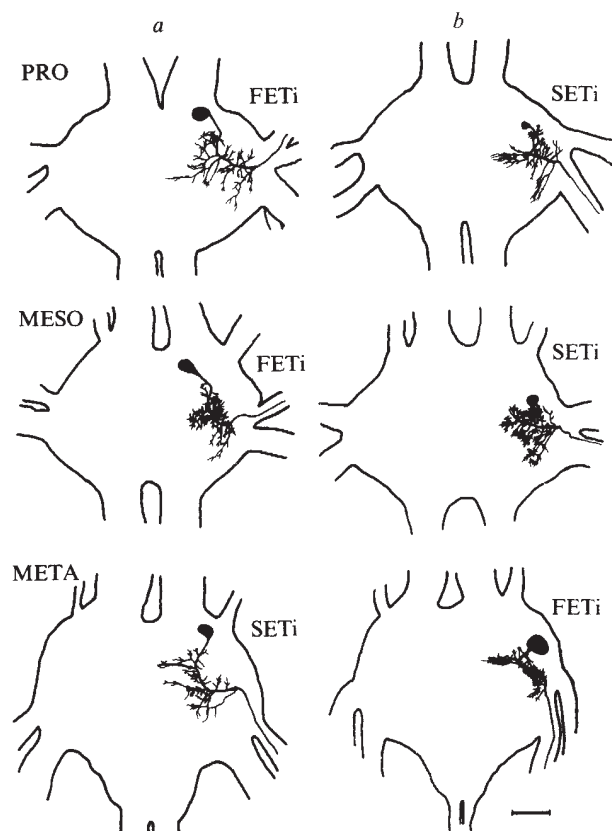
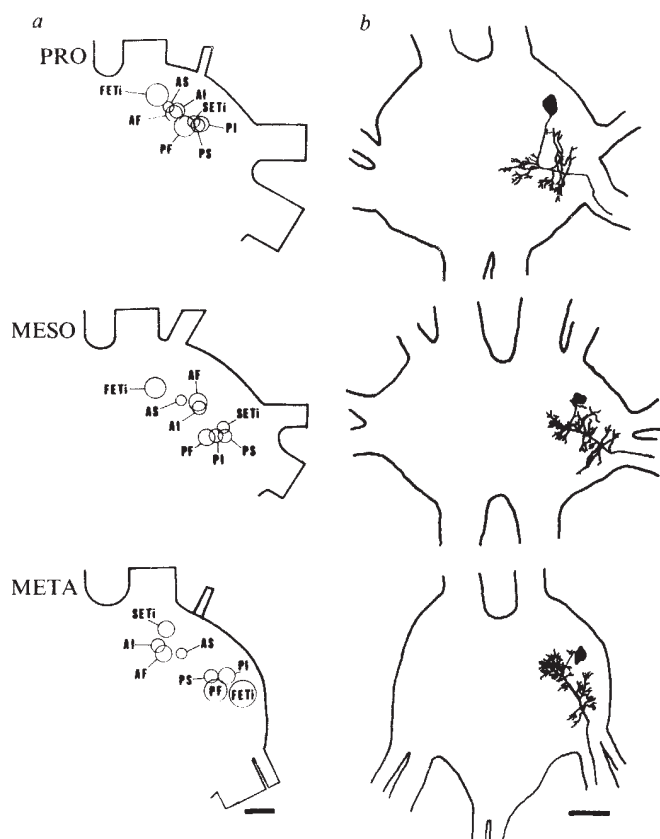


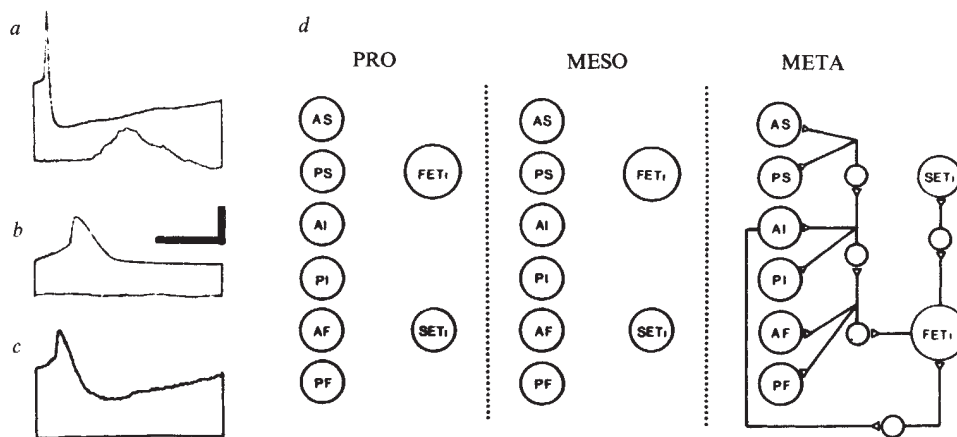
Fig. 2 Slow extensor tibiae (SETi) and fast extensor tibiae (FETi) motoneurones from camera lucida drawings of cobalt fills. The cells have been arranged such that serially homologous neurones are in columns *a* and *b* but analogous cells are not in the same columns, so as to illustrate the switch in functions in serially homologous neurones. Calibration, 200 μ m.

were identified using intracellular electrodes filled with either a Procion dye or cobalt chloride or nitrate. After physiological identification⁷, current of the appropriate polarity was passed to inject dye into the neurone. Ganglia were then processed using standard procedures⁸.

One hundred and fifty-one neurones innervating FITi and ETi neurones in the pro- and mesothoracic ganglia were studied in this way. Metathoracic FITi and ETi motoneurones, which had already been identified and stained with Procion dye⁵, were injected with cobalt in a similar way. Simultaneous penetrations of pairs of pro- and mesothoracic FITi and ETi neurones were made in 25 animals, and recordings from 88 FITi motoneurones were made while ETi motoneurone activity was monitored using myograms. Recordings from these experiments were then analysed to determine whether or not connections with latencies of less than 25 ms could be found between pro- and mesothoracic FITi and ETi neurones, as demonstrated for metathoracic FITi and ETi neurones⁷.

Six excitatory motoneurones which innervate the FITis in both the pro- and mesothoracic ganglia have been identified. The location of each cell body was determined using a computer graphics tablet with the appropriate programs⁹. Average locations of pro- and mesothoracic FITis were compared with the previously identified metathoracic FITis⁵. In each ganglion their cell bodies are positioned in an anterior and a posterior cluster, and in each cluster there is a 'slow' motoneurone, a 'fast' motoneurone, and one that causes an intermediate mechanical response⁷ (Fig. 1*a*). There are several morphological similarities for each particular type of FITi neurone in each of the thoracic ganglia. The cell body locations for each cell type are similar in all three ganglia. Each type of FITi has the same serially homologous neurones as neighbours;

Fig. 3 *a*, Simultaneous intracellular recordings from metathoracic FETi and AFFTi reveal the presence of a central connection between these neurones. AFFTi is seen to be depolarised after spikes in FETi in this averaged record of 16 spikes. *b*, Averaged record of 16 spikes in mesothoracic SETi and the response in PFFTi. No voltage change was seen in PFFTi; thus, no connection seems to be present between these cells. *c*, Averaged record of 16 spikes in mesothoracic FETi and the response in AFFTi. No voltage change is seen in AFFTi; thus, the absence of connections may be inferred. Calibrations 5 mV, 20 ms. *d*, Schematic summary of central connections between flexor and extensor tibiae motoneurons in the three thoracic ganglia. Flexor tibiae motoneurone abbreviations: AS, anterior slow; PS, posterior slow; AI, anterior intermediate; PI, posterior intermediate; AF, anterior fast; PF, posterior fast. No central connections were found between flexors and extensors in the pro- and mesothoracic ganglia, whereas in the metathoracic ganglion, FETi puts an e.p.s.p. on to all the flexors, AFFTi puts an e.p.s.p. on to FETi, and FETi puts an e.p.s.p. on to SETi. All these connections are thought to involve at least one interneurone^{8,11}.



for example, the anterior slow flexor (ASFITi) borders the fast depressor trochanter in each ganglion. All FITi motoneurons have equivalent variations in cell body locations between individuals. Camera lucida drawings of cleared whole mounts of ganglia containing cobalt-injected posterior fast flexor (PFFTi) motoneurons (Fig. 1*b*) show that the structure of the primary neurite is similar in each ganglion. Also, the neurite of PFFTi in each ganglion joins a tract which contains other serially homologous leg motoneurons, and dendritic branches occur in equivalent locations and depths in each of the three ganglia. We conclude that these neurones are serially homologous.

Examination of the two excitatory ETi motoneurons, however, has revealed a difference, for which we have a probable explanation. Pro- and mesothoracic fast extensor tibiae (FETi) and metathoracic slow extensor tibiae (SETi) axons are known to emerge in nerve 3, whereas pro and meso SETi and metathoracic FETi emerge in nerve 5 (ref. 9). Mapped cell body locations revealed that metathoracic FETi lies near the posterior flexors as do pro- and mesothoracic SETi cell bodies (Fig. 1*a*). An additional similarity between the metathoracic FETi and pro- and mesothoracic SETi is a pronounced loop that their axons make in the neuropile approximately 300 μ m before they enter the nerve trunk (Fig. 2). The metathoracic SETi and the pro- and mesothoracic FETi do not make such a loop. Their cell bodies are all located anterior to the serially homologous anterior flexors. Quantitative comparison of branching patterns has not proven possible; however, to a first approximation, pro- and mesothoracic SETi and metathoracic FETi have matching dendritic trees. The dendritic tree of metathoracic SETi matches those of pro- and mesothoracic FETi (Fig. 2).

Let us make the premise that the basic physiological properties determining slow and fast neuromuscular control are conservative and have been retained in evolution. This requires that in association with evolution of jumping the metathoracic FETi and SETi cell bodies switched positions, their axons switched nerve trunks, and their detailed branching patterns altered until they came to match those of their opposites in the other ganglia. That seems highly improbable. It is much more likely that a functional switch has occurred, the metathoracic SETi acquiring a fast role physiologically and the primaevial FETi a slow one without a change in cell body location, nerve trunk of exit, or branching pattern. Embryological evidence supports this view. Neuroblasts, with the exception of one medial neuroblast which arises late in the development of the prothoracic ganglion, are the same in number and arrangement

in each of the three thoracic ganglia¹⁰. This is an important conclusion for understanding the evolution of nervous systems, because it confirms the findings that neuronal morphology is conservative even when a dramatic functional change has occurred.

Next, we considered the sort of changes in the neural circuitry that may have been associated with the evolution of jumping. Central connections between the metathoracic flexor and extensor motoneurons have been described (Fig. 3*a* and *d*) and implicated in the neural control of jumping^{7,11}. They aid in locking the tibia into a flexed position and maintaining a co-contraction of FITis and ETis while force is built up and stored in the extensor muscle and the cuticle^{6,12,13}. We looked for comparable connections made in the pro- and mesothoracic ganglia by making simultaneous recordings in various pairs from SETi or FETi and one of the FITi Mns in the same ganglion. We confirmed the previous findings of connections between the metathoracic FETi and FITis^{7,10} (Fig. 3*a*). Significantly, no central cross-connection pathways were found between FITi and ETi neurones in the pro- or mesothoracic ganglia (Fig. 3*b* and *c*). This was true in 25 cases in which simultaneous intracellular recordings were made from an ETi neurone and a FITi neurone. Eighty-eight additional experiments, in which intracellular recordings from a FITi motoneurone were compared with myograms from the ETi muscle, further confirmed this finding.

In addition to the new central connections, major changes must have occurred in the periphery. Many new muscle fibres were added and these became innervated mainly by the transformed SETi axon that became FETi. These shifts may help understand the complexity of the innervation and muscle fibre population of the jumping muscle¹⁴. Through all these changes the primaevial neuronal geometry was highly conservative and retained.

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The units of calcium conduction in *Helix* neurones

VOLTAGE-DEPENDENT membrane calcium currents, I_{Ca} , occur in muscle^{1,2}, axons^{3,4} and nerve cell bodies^{5–9} but are not well characterised because of difficulties in separating them from other ionic currents, and inadequate spatial and temporal control of the clamp voltages used to examine them. As a result, information on the conduction process underlying I_{Ca} is limited. Recently two new techniques have been reported^{6,9,10} which overcome most of the problems associated with experimental investigations of I_{Ca} . We have used the suction pipette technique^{9,10}, which combines methods of internal perfusion and voltage clamp, to study I_{Ca} in isolated nerve cell bodies of the snail, *Helix aspersa*. The time course of I_{Ca} in response to step changes in membrane potential was readily examined and, in addition, very small fluctuations (noise) in current were also observed. Assuming that the fluctuations represent current contributions from a population of independent two-state (conducting/nonconducting) unit conductances, $\langle I_{Ca} \rangle$, the average value of the fluctuations in the steady state, and σ^2 , the variance of the fluctuations can be used to estimate a single unit conductance, γ_{Ca} . Analysis of spontaneous current noise has been used previously to obtain γ values for the Na and K systems in several axon membranes^{11–14}, and γ values for acetylcholine-sensitive conductances at the neuromuscular junction^{15,16} and in molluscan neurones¹⁷, but γ_{Ca} is unknown. Unit ion conductances of a few picosiemens or greater have been reported and where comparisons can be made, these values are similar to those estimated using quite different techniques such as tetrodotoxin binding^{18–21}. We found γ_{Ca} to be less than 1 pS however, and we suggest that the low value results from the effect of a Ca^{2+} binding site in the conductance.

Neurones from the circumoesophageal ganglia of *Helix aspersa* were exposed by removing the capsule of the ganglion. Part of a neurone of about 100 μ m diameter was sucked into the tip of a 50- μ m diameter suction pipette and removed from adjacent neurones and its own axon by pulling it free of the neuropil. A fine-tipped (5 μ m) platinised-platinum wire inside the pipette was advanced to puncture the neurone and gain access to the cell cytoplasm while maintaining an electrical isolation of 50–100 M Ω by the suction. The pipette^{9,10} also contained an agar bridge connection to a Ag:AgCl wire for measuring potential with respect to another agar bridge in the chamber solution coupled to another Ag:AgCl wire. An inlet tube, which is close to the tip of the pipette, allowed perfusion of the cell internally through the puncture. The standard internal solution consisted of 135 mM K aspartate adjusted to pH 7.4 with 28 mM Tris base. The extracellular solution was composed of the following: 85 mM NaCl, 5 mM KCl, 10 mM $CaCl_2$, 15 mM $MgCl_2$, 1 mM glucose and 5 mM Tris-HCl, pH 7.6 at room temperature.

The identification of I_{Ca} as a source of membrane current fluctuations was preceded by suppression of the other voltage-dependent ionic currents, I_K and I_{Na} . As reported previously^{9,10}, I_{Na} can be blocked either by Tris replacement of Na^+ or tetrodotoxin and I_K can be blocked by substituting Cs^+

for K^+ in the internal and external perfusates. The remaining currents are I_{Ca} and the leakage current. I_{Ca} may then be abolished by adding Ni^{2+} (1.0 mM) to the external solution leaving leakage current, which shows no voltage dependence at potentials below +150 mV.

A low noise voltage clamp system similar to that used by Fishman *et al.*²² was used. However, the isolated nerve cell body had an input resistance of 10 M Ω and a 100 M Ω feedback resistor, R_{fb} , was used to optimise the signal:noise ratio. Voltage control was not affected by R_{fb} and the dynamic response of the clamp was more than adequate for the noise frequencies of interest (< 125 Hz).

Analysis of current fluctuations in terms of power density spectra was done using a spectral analyser in a manner similar to that reported by Fishman *et al.*²². In addition, tape recorded fluctuations were reprocessed by digital computation using a fast Fourier transform program and difference spectra were calculated using a point by point subtraction of membrane current noise spectra before and after suppression of I_{Ca} with Ni^{2+} . The forms of the spectra were fitted with Lorentzian and/or f^{-1} functions using a modified Marquardt algorithm²³. The use of a Lorentzian function is reasonable since the kinetics of I_{Ca} are readily interpreted by a first order process of the Hodgkin-Huxley type²⁴, and the above model fits were consistent with this assumption. An f^{-1} function was added to test for the presence of substantial amounts of f^{-1} noise.

The variance calculated from the Lorentzian fit of a difference spectrum was also computed directly from the noise records before and after suppression of I_{Ca} . The estimate of variance in the latter method is model independent; that is, does not assume a first order process.

A third method for estimating the variance was also used. The fluctuation records were applied directly to a mean square meter, which consisted of a bandpass filter (1 Hz–1 kHz, 24 dB per octave, Butterworth), squaring circuit, timed integrator and d.c. voltmeter. All three methods were checked by applying gaussian white noise through a first-order low-pass filter and gave equivalent results.

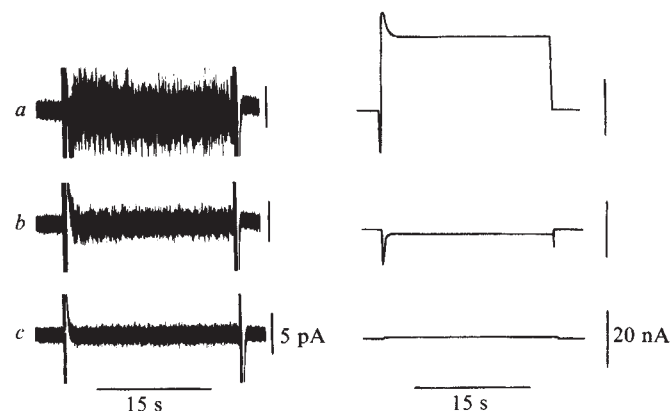


Fig. 1 *a*, Membrane currents before, during and after prolonged depolarisation of an isolated, internally-perfused, voltage-clamped snail neurone. Holding potential was -60 mV (potential of zero mean current) and the voltage was stepped to $+40$ mV. The left-hand panel shows the current noise traces and the large transients due to the high-pass filter; right-hand panels show the corresponding low gain recordings of membrane currents I_m after leakage correction. Current noise was analysed during the 15-s periods indicated by the horizontal bars while I_m was steady. Note the different current calibrations for the noise and I_m panels. *b*, After suppression of I_K with Cs^+ . Addition of tetrodotoxin or substitution of Tris for Na^+ had no further effect, and the current noise in (*b*) is attributed to fluctuations in I_{Ca} and leakage current. I_m was corrected for leakage and has transient and steady inward components. *c*, After addition of Ni^{2+} to the external solution. The difference in noise between (*b*) and (*c*) is attributed to suppression of I_{Ca} by Ni^{2+} . I_m is outward because of leakage.

The analysis of noise in the present experiments was done on 15-s blocks of membrane current data recorded after stepping the membrane potential from a holding value of -60 mV to $+40$ mV. Figure 1a shows current fluctuations and corresponding mean current before suppression of ionic currents. The beginning noise record in each case is the noise at the holding potential, followed by a transient response to the step change of potential, which results from the high-pass filter (corner at 0.5 Hz, single pole), followed by the current noise at the new potential. Noise analysis was carried out on the current fluctuations during the intervals indicated by the horizontal bars in Fig. 1. The mean current showed an initial inwards transient followed by an outwards current which also had a transient component similar to that described for molluscan neurones^{25,26}. Note, however, that the outwards current was steady while fluctuations were analysed. It is clear that the depolarising voltage step provoked a large increase in total membrane current noise. The contribution of I_K was then suppressed by Cs^+ , and as Fig. 1b shows, a depolarising step in voltage still provoked an increase in current noise. The mean

current now had a transient inward peak followed by a smaller inwards current which was steady while the fluctuations were analysed. Thus a significant portion of the current noise during a depolarising step is associated with I_K , but a substantial non- K^+ component remains. In these circumstances, hyperpolarising voltage steps of equivalent magnitude had no effect on membrane current noise levels. When Na^+ was replaced by Tris, or when tetrodotoxin was added to the external solution, the peak transient was reduced by about 30% but no consistent differences in either current noise or steady inwards current were observed. This is because inactivation of I_{Na} is complete at these long depolarisation times whereas in many neurones such as the present ones, a small steady inwards I_{Ca} , which is about 10% of the peak transient I_{Ca} , persists²⁷ (Fig. 1b). After addition of 1.0 mM of the I_{Ca} blocker, Ni^{2+} (ref. 24), the current fluctuations produced by depolarisation were greatly reduced and a small outwards leakage current was present in the mean current trace (Fig. 1c). As Ni^{2+} blocks I_{Ca} , the difference in current noise between b and c of Fig. 1 is attributed to fluctuations in I_{Ca} .

Further evidence for changes in the fluctuations of I_{Ca} after Ni^{2+} treatment comes from amplitude density analysis. Examples of the amplitude histogram computed directly from the membrane current noise records of Fig. 1b and c are shown in Fig. 2a and b. Although accurate determination of a gaussian distributed process requires information out to 6σ , which in turn requires long observation times, the histograms seem to have gaussian distributions. This is supported by the fact that the variance computed from the fluctuation record is equivalent to the variance calculated by assuming the amplitude density plot to be gaussian. The changes in σ after suppression of I_{Ca} are obvious.

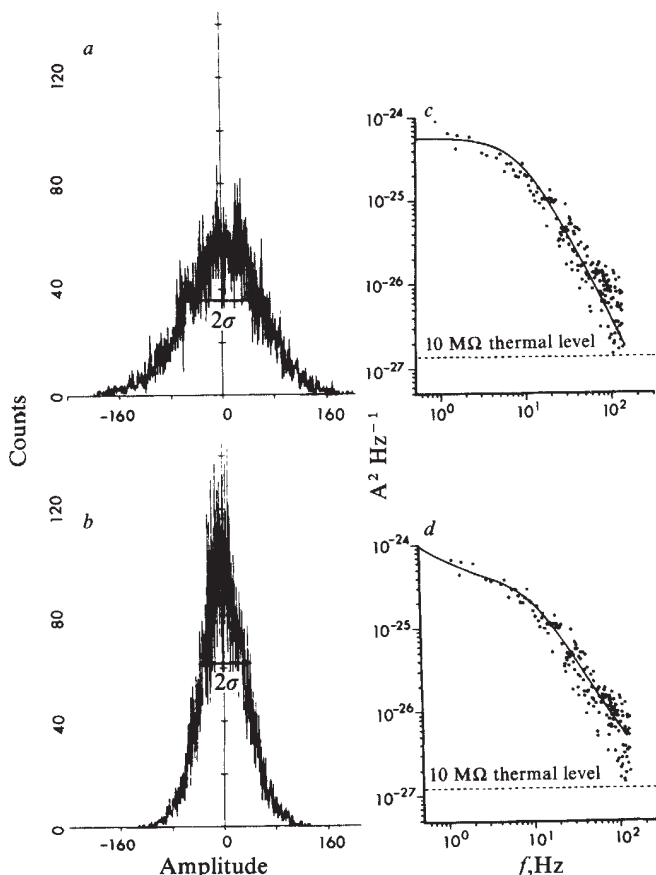
Difference spectra for records (b) and (c) of Fig. 1 are shown in Fig. 2c and d. The power densities of the spectra are two to three orders above the thermal noise ($\sim 10^{-27} \text{ A}^2 \text{ Hz}^{-1}$ corresponding to $10 \text{ M}\Omega$) of the neurone. The solid lines are for at least squares fit to the data points assuming in Fig. 2c a Lorentzian function of the form $K_2[1 + (f/f_c)^2]^{-1}$ where f_c is the corner frequency (model 1) or assuming in Fig. 2d a $K_1 f^{-1}$ plus Lorentzian function (model 2). The χ^2 values for models 1 and 2 are the same, 1.2×10^{-3} , so that the data do not permit discrimination between the two models. The functions being fitted, however, are not arbitrary since the fit of the sum of two Lorentzian functions (model 3) to the data, or the sum of two Lorentzian functions plus f^{-1} (model 4), had notably larger χ^2 values and an f^{-1} function (model 5) did not fit the data well at all. In one experiment, better fits were obtained when several 15-s noise records were averaged, as would be expected, but models 1 and 2 still provided noticeably better fits.

A two-state unit conductance may be calculated from the spectra using the relation

$$\gamma_{\text{Ca}} = \frac{S_1(0) \frac{\pi}{2} f_c}{\langle I_{\text{Ca}} \rangle (V - V_{\text{Ca}}) (1 - a)} \quad (1)$$

where $S_1(0)$ is K_2 , the low frequency asymptote of the Lorentzian component of the fitted spectrum, and f_c is the corner frequency. $\langle I_{\text{Ca}} \rangle$ is the mean current at long times and had a typical value of 2 nA . V is the membrane potential ($+40 \text{ mV}$), V_{Ca} is the potential at which there is no current flow for Ca^{2+} (between 140 and 180 mV) and a is the ratio of ion conductance, g_{Ca} , measured in the steady state at V to its maximum attainable value, $\bar{g}_{\text{Ca}}$. This ratio can be estimated from the ratio of steady and peak transient I_{Ca} values (2 and 20 nA respectively). A chord conductance of $2 \times 10^{-7} \text{ S}$ was calculated from peak I_{Ca} at $+40 \text{ mV}$ and was about 95% of $\bar{g}_{\text{Ca}}$. In all instances the value of a was 0.1 or less and was therefore ignored. The low values result from the fact that most of the conductance sites were inactivated while the current fluctuations were being analysed. Thus at $+40 \text{ mV}$, the activation time constant for I_{Ca} , τ_m is less than 1.0 ms (refs 24, 27) whereas there are two inactivation time constants, τ_{h1} and τ_{h2} in the present neurones

Fig. 2 Analysis of I_{Ca} fluctuations shown in Fig. 1b and c using amplitude histograms and spectral power-density plots. The instantaneous amplitude of a fluctuation sample record was measured and sorted to give amplitude histograms. The variance computed directly from the sample record and from the amplitude histogram assuming a gaussian distribution were the same. The scale on the abscissa is arbitrary. a and b are before and after suppression of I_{Ca} with Ni^{2+} and the reduction in variance is apparent. Difference spectra for (b) and (c) of Fig. 1 are shown in (c) and (d). The spectra are based on 7,500 digital samples taken at 500 Hz through a 250-Hz active low-pass filter. The solid lines are the least squares fits using the Marquardt algorithm with the expression $K_2[1 + (f/f_c)^2]^{-1}$ in (c) and $K_1 f^{-1} + K_2[1 + (f/f_c)^2]^{-1}$ in (d), where f_c is the corner frequency. The fits were equivalent using either function. The calibration for a $10\text{-M}\Omega$ resistor is indicated by the dashed lines.



with values of about 10–15 ms and 250–350 ms respectively²⁷. Most of the transitions are therefore probably from open to inactivated states rather than from closed to open states. In this regard it is of interest that no additional spectral components were observed by extending the recording bandwidth from 1.0 to 0.1 Hz at low frequencies or from 125 Hz to 1 kHz at the high frequencies.

Calculations of γ_{Ca} yield estimates on the order of 6×10^{-13} to 6×10^{-14} S, the exact value depending on the model used to fit the data (Table 1). In two experiments, model 2 was the best fit, and in another experiment, models 1 and 2 gave equally good fits (Table 1). The γ values using either model are similar. The model independent variances, σ_i^2 , do not discriminate between f^{-1} and f^{-2} noise sources, but gave values similar to those calculated from the spectral fits. Thus the unit conductances, calculated as $\sigma_i^2/\langle g_{Ca} \rangle$ where $\langle g_{Ca} \rangle$ is the mean Ca^{2+} conductance for a given potential, also were similar among the three methods, which suggests that the noise being measured is mainly of the f^{-2} type.

The corner frequency for the Ca^{2+} conductance determined from its power spectrum ranges from 10 to 20 Hz which is low compared to the turn-on kinetics of I_{Ca} , but which as we have noted is appropriate for the faster inactivation process^{24,27}. Humps related to the slower inactivation process and the high frequency activation process were not evident in our records probably because the number of relevant transitions was too small in both cases.

The values for γ_{Ca} are smaller than values reported for axonal unit conductances for Na^+ and K^+ , but there are no comparable measurements on somal membranes. Therefore, we compared the values of unit conductances activated by acetylcholine (ACh) applied to the somal membranes with values reported for other molluscan neurones¹⁷. The details of these experiments will be reported elsewhere. Briefly we found that ACh produced excitatory or inhibitory responses as expected and large increases in membrane current noise. Difference spectra were computed from ACh and control records and were fitted with single Lorentzian functions similar to those shown in Fig. 2c and d. Unit conductances of 10^{-11} to 10^{-12} S and time constants of 10–15 ms were calculated; these values are very similar to those reported for ACh-activated conductances in *Aplysia* neurones¹⁷. Thus, γ values associated with voltage-dependent Ca^{2+} conductances are smaller than ACh-activated γ values in the same somal membranes and are smaller than currently reported γ_{Na} or γ_K values. Low values for γ_{Ca} may also be present in the presynaptic axons of the squid stellate synapse using calculations based on $\langle I_{Ca} \rangle$ and density of presumed transport sites²⁸. The density of Ca^{2+} conductance sites in snail neurones may be calculated from values of $\bar{g}_{Ca}$ assuming the cell body to be a sphere 100 μm in diameter¹⁰ and has values of 10–100 μm^{-2} which are within the range reported for Na^+ and K^+ conductance units in squid axon^{29,30}.

An obvious question is whether there is anything about the Ca^{2+} conductance that might be responsible for these low γ_{Ca}

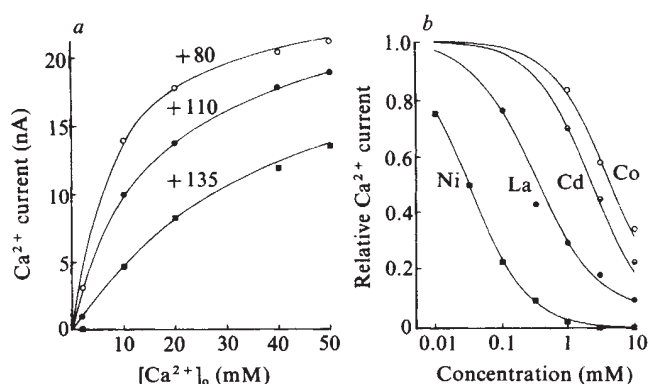


Fig. 3 Evidence for a site in the calcium conduction process that binds Ca^{2+} and other divalent and trivalent cations. In (a) the peak inward Ca^{2+} current, after leakage correction, elicited by a voltage step from -60 to the voltage indicated was plotted against various $[Ca^{2+}]_o$ values. The voltage clamp Ca^{2+} currents referred to are shown in refs 10 and 24. The solid lines are computed from Langmuir adsorption theory using

$$I_{Ca}(V) = \frac{I_{Ca} \max(V)}{1 + K_{Ca}(V)/[Ca^{2+}]_o} \quad (2)$$

where I_{Ca} is the peak transient current at a given voltage, V , $I_{Ca} \max(V)$ is I_{Ca} when all the sites are occupied by Ca^{2+} and $K_{Ca}(V)$ is the dissociation constant of the binding site. Theory fits well except at low $[Ca^{2+}]_o$ values which may not be accurate because Ca^{2+} buffers were not used. Binding of Ca^{2+} seems to be voltage dependent. In (b) the effects of several multivalent cations added to the external solution at different concentrations are shown. Relative Ca^{2+} current refers to the ratio of I_{Ca} values before and after addition of a particular ion. The solid lines are theoretical fits to a modification of equation (2) used by Hagiwara³¹ to calculate the different ionic dissociation constants of the site.

values. The answer may be provided by two important properties of I_{Ca} identified in these neuronal membranes²⁴ and in barnacle muscle³¹. These are that I_{Ca} saturates over a small range of external Ca^{2+} concentrations $[Ca^{2+}]_o$ (Fig. 3a) and that I_{Ca} is blocked by divalent cations such as Cd^{2+} , Co^{2+} and Ni^{2+} and the trivalent cation La^{3+} (Fig. 3b). The results can be fitted by a Langmuir adsorption function and may be attributed to a site in the conduction process that adsorbs divalent and trivalent cations. In snail neurones the site seems to be voltage dependent, binding Ca^{2+} less strongly at more depolarised voltages (Fig. 3a). Adsorption of Ca^{2+} by a channel site could readily retard the transport of Ca^{2+} across the plasma membrane and result in low unit conductance values such as those reported presently. An alternative to restricted diffusion would be transport of Ca^{2+} by a carrier but this seems less likely since the noise levels reported presently are too high and the power spectrum is not consistent with those reported for carriers³².

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Table 1 Comparison of unit conductances in Siemens, calculated from variances determined from amplitude histograms or from spectral fits using the models referred to in the text

Expt	$\gamma_{A.H.}$ (S)	γ_1 (S)	f_{c1} (Hz)	γ_2 (S)	f_{c2} (Hz)	$\langle I_{Ca} \rangle$ (nA)
10	1.1×10^{-13}	1.0×10^{-13}	12.0	0.6×10^{-13}	17.1	2.0
14	1.3×10^{-13}	5.6×10^{-13}	18.0	6.2×10^{-13}	11.4	2.5
6	1.0×10^{-13}	1.4×10^{-13}	20.5	1.8×10^{-13}	15.6	1.5

$\gamma_{A.H.}$ is calculated from the amplitude histogram; γ_1 from a model 1 fit to the spectral plots and γ_2 from a model 2 fit to the spectral plots. f_{c1} and f_{c2} are the corner frequencies (Hz) for the respective models. $\langle I_{Ca} \rangle$ is the value in nA for the steady inward current while the fluctuations were being analysed.

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Characterisation of a synthetic, voltage-dependent, cation-selective transmembrane channel

THE limitations of the gramicidin channel as a model for physiological channels are the apparent requirement of D-amino acids in the channel structure and the absence of a useful voltage dependence. The synthetic channel, HCO-(L-Ala-L-Ala-Gly)₅-OMe, is shown here to form monovalent cation-selective channels in planar lipid bilayers where they exhibit a significant voltage dependence. The selectivity is similar to that of gramicidin; but the channel mean lifetime and voltage dependence are dramatically different, making the synthetic channel more similar to physiological channels.

Derivation of the synthetic channel, some years ago, utilised a set of conformational arguments which were based on a new concept referred to as cyclic conformations with linear conformational correlates^{1,2}. This analysis led to a set of linear, helical structures which could form two types of helices with a change in dipole moment of the order of 3 D Å⁻¹ along the helix axis on interconversion between two conformations. One of the helical structures contained a gramicidin-like channel and was called a β_{2,4}-helix and the other contained a β-turn repeating on a helix axis and was called a syn-β₂⁶-spiral. The primary structural requirement was the sequence (L-amino acid-L-amino acid-Gly)_n. Simple analogues, HCO-(L-Ala-L-Ala-Gly)_n-OMe with *n* = 4 and 5, were synthesised and found to form channels with conductances similar in size to those of gramicidin; and the channels could be seen to turn on and off with sweeping of voltage^{3,4}. Previously, the demonstration of an exponential voltage dependence for the number of channels formed was not possible, presumably due to the fragility of the planar lipid bilayer, and the channels formed were not characterised as to mean lifetime, nor were they checked for monovalent compared with divalent cation selectivity. Exclusion of divalent cation is necessary for a gramicidin-like channel, where the channel axis is coincident with the helix axis and only a single thickness of a polypeptide chain shields the cation in the channel from the low dielectric constant of the lipid layer⁵. An alamethicin-like channel, on the other hand, is a barrel-stave alignment of structures, probably helices, providing a thicker structure separating a relatively non-selective channel from the lipid^{6,7}.

Bilayers were formed using 2% (w/v) diphytanoyl-L-α-lecithin in *n*-decane using a previously described method and

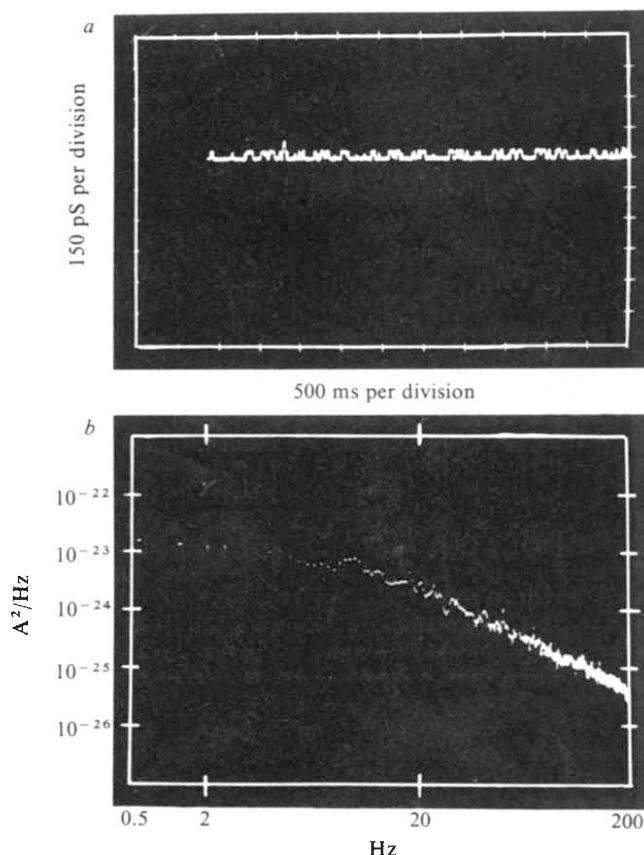
apparatus⁸ with a 2-mm aperture. After the membrane had become black for 1 h or more, the synthetic peptide was added to the aqueous bath on one side of the lipid bilayer as a trifluoroethanol solution containing about 5% (v/v) trifluoroacetic acid. The latter was present to assist solubilisation of the synthetic peptide, HCO-(L-Ala-L-Ala-Gly)₅-OMe. The same quantity of solvent was checked separately and found to have no effect on the conductance properties of the lipid bilayer.

Figure 1 shows single-channel fluctuations. The spectrum shown in Fig. 1b could be approximated by a single Lorentzian function of the form

$$S(f) = \frac{2\gamma\mu\tau}{1 + (2\pi f\tau)^2} \quad (1)$$

where *f* is frequency; *μ* is mean membrane conductance; *τ* is the mean lifetime of the channel; and *γ* is the single channel conductance. The single-channel conductance for 1 M CsCl at 25 °C and 50 mV was 51 pS and was found to be ohmic, that is, the single channel currents were linear with voltage. The mean channel lifetime (*τ* = 1/2*πf_c*) is calculated from the power spectrum with *f_c* defined as the half-power frequency. The mean channel lifetime is about 30 ms. This is a very rapid channel similar in magnitude to that of physiological channels and quite distinct from that of gramicidin with *τ* > 1 (ref. 9). Addition of 0.1 M CaCl₂ resulted in little or no activity. Thus, although the synthetic channel exhibits similar unit channel conductance and non-permeability to calcium ion, the mean channel lifetime differs by one or two orders of magnitude from that of gramicidin^{9–12}.

Fig. 1 *a*, Single-channel fluctuations recorded on a storage oscilloscope after current amplification (10¹⁰) and low pass filtering (1,000 Hz). *b*, Power spectral density of bilayer current fluctuations induced by a 100 pM concentration of the synthetic peptide. Spectrum was computed from the fast Fourier transform of 1,024 points, digitised from a 2-s epoch and averaged over 32 epochs. Recording conditions as in *a*.



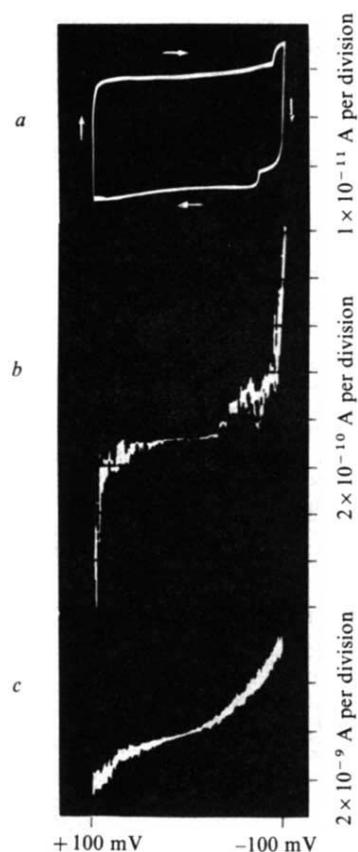


Fig. 2 Voltage dependence examined by sweeping voltage in a triangular wave (0.1 Hz). Current is measured after amplification using a Keithley 427 current amplifier.

Voltage dependence is shown in Fig. 2. In Fig. 2a, a single channel is seen to turn on at about 90 mV (top left) and to turn off (lower left) as the voltage returns below 100 mV. For Fig. 2b, 100 pM of synthetic peptide was added to the bath and a large number of channels (several hundred) are seen to be on at ± 100 mV with almost no channels in the ± 30 mV range. (The capacitance step on reversing potential is no longer seen due to the change of vertical scale.) In Fig. 2c with an even greater number of channels in the membrane (note again the change in scale), the exponential dependence of conductance on voltage is apparent.

The free energy change for channel formation, ΔF , is given by

$$\Delta F = \frac{\Delta\mu}{e} FE \quad (2)$$

where $\Delta\mu$ is the effective dipole moment change for channel formation; F is the Faraday constant of 23 kilocalorie V^{-1} ; E is the electric field in $V\text{ cm}^{-1}$; and e is the unit electronic charge of 4.8×10^{-10} e.s.u. As the field is applied over the thickness of the lipid layer, $E = V/l$, where l is the thickness of the lipid layer of the planar bilayer. The quantity of interest is $\Delta\mu$ per $\text{\AA}$ of lipid layer. As the channels are ohmic, it is possible to calculate from Figs 2b and c the number of channels at 50 mV and at 100 mV and to plot the log of the number of channels against voltage. The slope of the plot allows calculation of $\Delta\mu$ per $\text{\AA}$. The calculated values for the positive and negative sweep directions from Figs 2b and c give values ranging from 2.6–4 D $\text{\AA}^{-1}$. The conformational analysis yields an expected value of greater than 3 D $\text{\AA}^{-1}$ when neglecting end-group effects¹³.

Thus, this first cation-selective, synthetic channel, which contains no D-amino acids, and while exhibiting single channel

conductances and ion selectivity similar to gramicidin, exhibits a striking voltage dependence and mean lifetimes which are similar to those of physiological channels^{14,15}. It is perhaps worth reiterating that the proposal for synthesis and for testing of the polypeptide in lipid bilayers was the conformational analysis called cyclic conformations with linear conformational correlates which was utilised to derive a molecular theory of electric field-dependent interconversion between conducting and non-conducting conformations. Extensive conformational studies on the cyclic conformations and their linear conformational correlates are presently underway.

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GABAergic modulation of benzodiazepine binding site sensitivity

ALTHOUGH neurophysiological, behavioural and biochemical evidence suggests that benzodiazepines (BZ) may affect several neuronal systems within the central nervous system (CNS), recent studies indicate that some of these effects result from a specific interaction with GABAergic transmission¹. The precise mechanism of this interaction remains in doubt, as facilitation of GABAergic transmission², potentiation of GABAergic inhibition^{3,4}, direct activation of γ -aminobutyric acid (GABA) receptors⁵, and antagonism of GABA-mediated inhibition have all been attributed to the benzodiazepines^{6,7}. In studies from this laboratory⁸, both the systemic and iontophoretic administration of BZs potentiated an inhibitory response produced by GABA in the dorsal raphe nucleus—suggesting that the potentiation was mediated through a change in the post-synaptic receptor. In addition, direct binding studies using ³H-diazepam have indicated a specific high affinity binding site which may be relevant to the pharmacological actions of BZ in brain⁹. In this study, we show that GABA can modulate the responsiveness of this BZ binding site since the addition of GABA to cortical membranes *in vitro* results in an increased affinity of the ³H-diazepam binding site for its ligand. This effect is mimicked by the GABA analogue, muscimol¹⁰, and antagonised by the GABA antagonist, (+)bicuculline.

Using washed membranes prepared from rat cortex, we demonstrated an enhancement of specific ³H-diazepam binding when the membranes were incubated with either GABA or the GABA agonist, muscimol (Table 1). This effect was entirely

reversed by the cortically active GABA antagonist, (+)-bicuculline methiodide¹¹ (the chemically stable form of this drug¹²); (+)bicuculline methiodide also decreased the binding of ³H-diazepam below control levels. (-)Bicuculline methiodide which is a relatively inactive isomer¹³ at the GABA receptor was less active than (+)bicuculline methiodide in reversing the GABA enhancement of binding. In contrast, picrotoxin which has been shown to be inactive in electrophysiological experiments in cortex¹⁴ was unable to alter specific ³H-diazepam binding of cortical membranes. A series of putative amino acid transmitters and related compounds was also tested for effects on ³H-diazepam binding. Only GABA and muscimol were active in this series (Table 2).

The increase in ³H-diazepam binding in the presence of GABA and muscimol was dose-dependent (Fig. 1) and half-maximal effect was obtained at 1.6 μ M for GABA (significantly different ($P < 0.05$) from control at $> 1 \times 10^{-6}$ M) and 0.5 μ M for muscimol (significantly different from control at $> 1 \times 10^{-7}$ M and significantly higher than GABA at 5×10^{-6} M of each drug, $P < 0.05$). (+)Bicuculline methiodide reversed the effect of 10 μ M GABA and could decrease control binding; the observed half-maximal effect was 5.0 μ M for the combined inhibition of the increase in ³H-diazepam binding due to 10 μ M GABA and control binding. If (+)bicuculline is a competitive inhibitor of GABA receptors (which is controversial¹⁵), this would correspond to a K_i of 0.7 μ M. Consistent with this view, (+)bicuculline elicited a half-maximal inhibition

Table 2 Effect of putative amino acid transmitters on ³H-diazepam binding *in vitro*

Compound	% Control binding
Control	100 $\pm$ 2
GABA	125 $\pm$ 3*
Muscimol	133 $\pm$ 3*
DABA	102 $\pm$ 1
Aspartate	104 $\pm$ 2
Glutamate	103 $\pm$ 2
Glycine	104 $\pm$ 2
Proline	106 $\pm$ 2
Taurine	101 $\pm$ 3
Aminooxyacetic acid	98 $\pm$ 3
Imidazole acetic acid	97 $\pm$ 4

Assays were carried out as described in Table 1 and in the presence of 10^{-5} M concentration of each of the above compounds. Results of several separate experiments have been combined and per cent compared to each control is reported. For each drug, $n = 8$. Values are means $\pm$ s.e.m.

* Different from control ($P < 0.001$).

of control binding at 1 μ M (data not shown) which is similar to the calculated K_i . The data presented in Table 1 also indicate a stereospecific difference of at least 25-fold between (+) and (-)bicuculline effects on GABA-activated binding since the lower limit of the K_i for (-)bicuculline is 15 μ M.

The increase in specific ³H-diazepam binding is due almost entirely to shifts in the affinity of ³H-diazepam for its receptor (Fig. 2). In control membranes, the apparent K_D for ³H-diazepam was 4.1 nM and the total number of sites was 433 fmol per incubation. In the presence of GABA (1×10^{-5} M), this K_D was shifted to 2.3 nM but the maximal number of sites was not altered. Thus, the apparent affinity but not the number of receptor sites is altered by the interaction of GABA and the membranes. (+)Bicuculline methiodide lowered the apparent affinity for ³H-diazepam ($K_D = 6.4$ nM) without a change in the number of sites. In the case of muscimol, a major increase in affinity was noted ($K_D = 1.9$ nM) along with a small change in maximal binding. These changes in apparent K_D for ³H-diazepam binding are due to changes in the association rate constant [k_1 (control) = $3.8 \pm 0.3 \times 10^7$ M⁻¹ min⁻¹; k_1 (GABA

Table 1 Effect of GABA and related drugs on ³H-diazepam binding

Drug	Specific ³ H-diazepam binding (c.p.m. $\pm$ s.e.m.)
Expt 1, 2 nM ³ H-diazepam	
None	6,513 $\pm$ 139
GABA (5 μ M)	7,991 $\pm$ 107*
(+)Bicuculline methiodide (100 μ M)	4,079 $\pm$ 90†
GABA + (+)bicuculline methiodide	3,949 $\pm$ 41‡
Muscimol (10 μ M)	8,657 $\pm$ 78*
GABA + muscimol	8,534 $\pm$ 118
Muscimol + (+)bicuculline methiodide	
GABA + picrotoxin (10^{-5} M)	
Expt 2, 1 nM ³ H-diazepam	
Control	3,839 $\pm$ 61
GABA (10 μ M)	4,760 $\pm$ 67†
GABA + (+)bicuculline methiodide (100 μ M)	3,347 $\pm$ 55*,‡
GABA + (-)bicuculline methiodide (100 μ M)	4,424 $\pm$ 136*

Male Sprague-Dawley/ARS rats (approximately 220–250 g) were decapitated, their brains were removed and the cortex was immediately dissected. The cortex was homogenized in 100 vol (w/v) of 0.05 M Tris-HCl buffer (pH 7.5 at 4°C) using a Brinkmann polytron. The homogenate was centrifuged at 20,000g for 15 min, the pellet was resuspended in another 100 vol of buffer and repelleted. The pellet was resuspended in 10 vol of the same buffer. Typical incubations contained in a total volume of 500 μ l, 100 μ l of cortical membranes (approximately 0.5 mg protein), 2 nM ³H-diazepam (39.1 Ci mmol⁻¹, New England Nuclear Co.) and the drugs under study (Experiment 1). Incubations were carried out for 30 min at 4°C. After incubation the samples were mixed, filtered under low vacuum through GF/B filters (Whatman Co.) and the filters rinsed twice with 7.0 ml of the same Tris buffer. The filters were dried and counted in a liquid scintillation counter. Specific ³H-diazepam binding is defined as total ³H-diazepam binding minus binding obtained in the presence of 10^{-5} M chlordiazepoxide. With 2 nM ³H-diazepam, nonspecific binding was approximately 950 c.p.m. and nonspecific binding did not vary with any of the treatments reported in this paper.

* $P < 0.01$; $n = 4$ compared to control.

† $P < 0.001$; $n = 4$ compared to control.

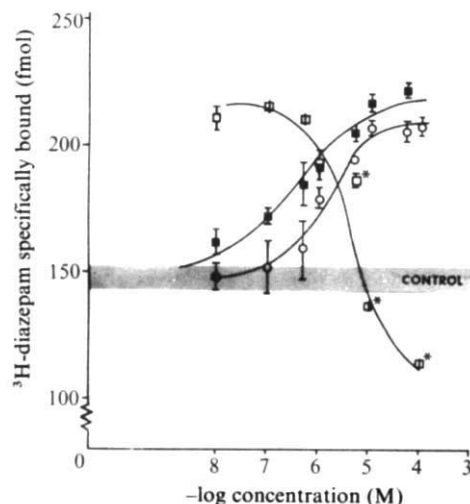
‡ $P < 0.001$; $n = 4$ compared to GABA alone.

§ $P < 0.001$; $n = 4$ compared to muscimol alone.

|| Not different from GABA.

¶ $P < 0.001$; $n = 4$ compared to GABA + (+)bicuculline methiodide.

Fig. 1 Effect of varying concentrations of GABAergic drugs on ³H-diazepam binding. Cortical membranes were prepared and assayed for ³H-diazepam binding according to Table 1 and the concentration of each drug ($n = 4$) was varied as indicated. The shaded area represents control binding ($\pm$ s.e.m.) in the absence of added GABAergic drugs. ○, GABA; ■, muscimol; □, bicuculline + GABA. * $P < 0.01$ compared to 1×10^{-5} M GABA.



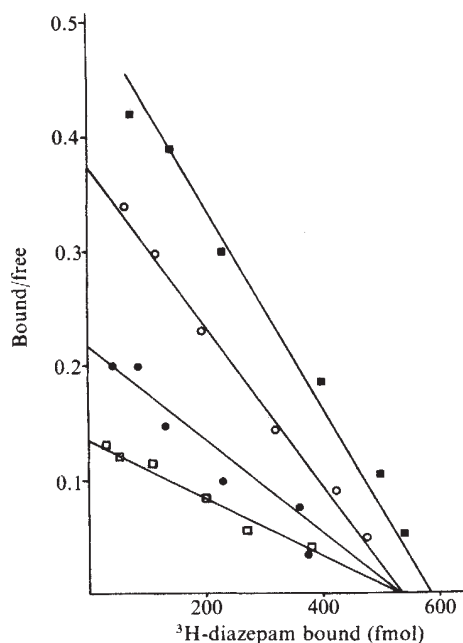


Fig. 2 Scatchard analysis of ^3H -diazepam binding in the absence of added drug (●) or in the presence of GABA (1×10^{-5} M) (○), muscimol (1×10^{-5} M) (■) or (+)buciculline methiodide (1×10^{-4} M) (□). Assays were carried out as described in Table 1 except that the concentration of ^3H -diazepam was varied as indicated.

treated) = $7.1 \pm 0.4 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$, $P < 0.01$]; the dissociation rate constant was unaltered ($k_2 = -0.122 \pm 0.02 \text{ min}^{-1}$) [data not shown].

The results reported here support an intimate interaction between GABA and BZ in brain. As directly shown using cortical membranes *in vitro*, GABA and its analogue muscimol can increase the affinity of the BZ for its binding site resulting in greater binding of low doses of BZ to its 'receptor' (Table 1). A similar situation has been observed *in vivo*¹⁶ where an increased binding affinity for BZ occurs after pretreatment of rats with AOAA which increases endogenous GABA levels. In addition, the ability of (+)buciculline to decrease the affinity of the diazepam receptor for BZ below untreated control levels suggests that the control state of the receptor may already be somewhat activated, perhaps due to a postdecapitation elevation of GABA levels¹⁷. Note that at the concentrations used in this study (+)buciculline does not interfere with ^3H -diazepam binding to a solubilised and partly purified receptor (J.F.T., unpublished) which rules out an alternate interpretation that there is a direct interaction at the binding site.

The specificity of GABA in enhancing the affinity of the BZ receptor is demonstrated by the inactivity of a series of putative amino acid transmitters (Table 2). In particular, taurine, glycine and proline which are inhibitory transmitters were inactive indicating chemical specificity for the inhibitory transmitter GABA; DABA, an inhibitor of presynaptic GABA uptake sites¹⁰, was also inactive indicating a probable postsynaptic site of action of GABA. This suggestion is also supported by our evidence that (+)buciculline is more potent than (-)buciculline indicating a synaptic site of GABA action¹⁸ and evidence that indicates most of the GABA receptors in cerebral cortex are postsynaptic¹⁹. Interestingly, imidazole acetic acid which is a potent inhibitor of ^3H -GABA binding¹⁸ was inactive in our system. However, electrophysiological studies have suggested²⁰ that the receptor sites for imidazole acetic acid and GABA may have differences.

It is important to note that this system seems to be one of the few where a receptor affinity change rather than a change in the number of binding sites²¹⁻²³ mediates an alteration in physio-

logical sensitivity. Such a change in affinity might be expected if the GABA receptor were directly coupled to a BZ receptor, or if GABA were an allosteric activator of a BZ receptor, or if the BZ binding site is part of a GABA/BZ/ionophore complex. Our results indicate that GABA can affect this binding site *in vitro* suggesting an alteration in its conformation. Thus, the interaction between GABA and BZs in brain provides a mechanism for the pharmacological modulation of a known transmitter system. The existence of an endogenous benzodiazepine ligand which may also act at the BZ binding site remains a possibility, but we feel that the interaction between GABA and the BZ binding site described here is sufficient to explain many of the electrophysiological effects of the BZs^{2-4,8}. However, whether such an interaction could account for all of the pharmacological effects of the BZs in the CNS is not yet known.

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Overcoming conduction failure in demyelinated nerve fibres by prolonging action potentials

LOSS of myelin from central or peripheral myelinated nerve fibres results in conduction delays, increased refractoriness and, above all, conduction block^{1,2}. Conduction failure could result either because a demyelinated axon is electrically inexcitable, or because the limited current available from a normal node is insufficient to excite it. Because continuous conduction along demyelinated internodes has been observed³, it seems likely that demyelinated axons in general are able to conduct impulses, but that the impedance mismatch at the junction of normal and demyelinated axon usually prevents the realisation of this ability. In support of this, two factors have been shown to improve transmission in experimental demyelination and also, transiently, in multiple sclerosis patients: lowered calcium

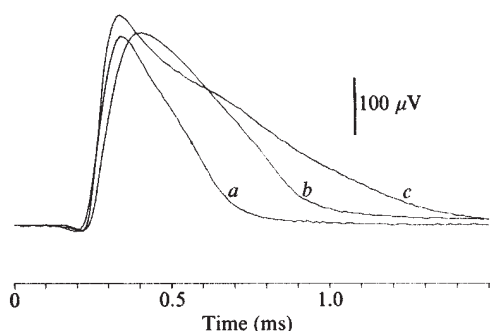
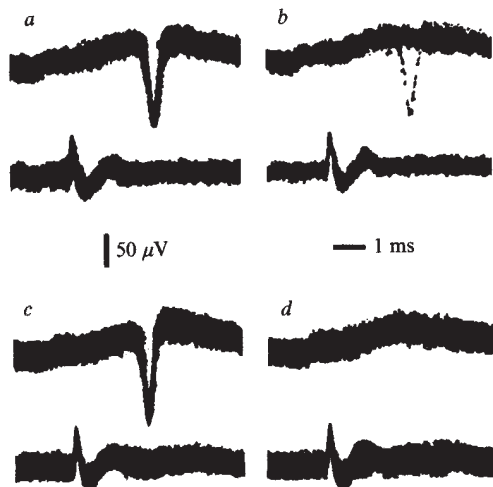


Fig. 1 Action potentials recorded from single fibre in rat ventral root at 37°C (a), at 31°C (b), and at 37°C after exposure to scorpion venom for 30 s (c). Each trace is average of 512 sweeps.

ion concentration^{4,5}, which reduces the threshold for nerve excitation, and lowered temperature^{6,7}, which prolongs the action potential and thereby increases the time integral of current available to depolarise and excite the demyelinated axon. To test the suggestion that a drug that specifically prolongs the action potential might prove useful in the therapy of demyelinating diseases⁸, we have compared the effects of temperature and a scorpion venom on transmission in single fibres through a demyelinating lesion in rat spinal roots.

By analogy with squid and amphibian nerves, we expected that mammalian nerve action potentials would be prolonged both by substances that reduce potassium current and by those that slow the inactivation of sodium current. In frog fibres tetraethylammonium (TEA) ions selectively and reversibly abolish potassium current⁹ and 5 mM TEA prolongs action potentials threefold¹⁰. However, we found TEA ineffective in prolonging action potentials in rat, even at a concentration of 60 mM, suggesting that action potential duration does not depend on potassium current in these fibres. In frog fibres, the venom of the scorpion *Leiurus quinquestriatus* acts primarily by delaying sodium inactivation¹¹ and prolongs action potentials irreversibly by 1,000 times or more¹². We found a similar effect on rat fibres, as shown in Fig. 1. This compares the action of the venom and reduced temperature on a single fibre action potential in a rat ventral root. Whereas both treatments prolong the action potential, the venom primarily affects the falling

Fig. 2 Action potentials recorded either side of chamber in which temperature was altered and scorpion venom applied. In each part the lower trace was recorded biphasically before the chamber, and the upper trace monophasically after it. a, 26°C and b, 26.7°C before venom; c, 32.5°C and d, 32.9°C after venom applied for 30 s. In each part the responses to about 20 stimuli are superimposed.



phase and has little effect on conduction velocity. Exposure for several minutes to the same concentration ($2.5 \times 10^{-5} \text{ g ml}^{-1}$ crude lyophilised venom from Sigma) results in almost rectangular action potentials, tens of ms in duration, as reported for amphibian fibres¹².

To compare the effects of venom and temperature on impulse transmission, a rat spinal root preparation was used. A focal demyelinating lesion was induced with diphtheria toxin as previously described³, and 6–8 d later, in a terminal experiment under urethane anaesthesia, the roots were exposed by an extensive laminectomy. A root was cut rostrally, threaded through a small Perspex chamber and sealed in with soft paraffin wax. The temperature of the 4.0-mm length of root within the chamber was monitored with a thermistor and altered by heating or cooling the Ringer solution perfusing the chamber, while the rest of the root was maintained at constant temperature in a pool of oil. A single fibre in the root was excited by electrical stimulation in the tail and its action potential was recorded on either side of the chamber while the temperature of the Ringer solution was varied.

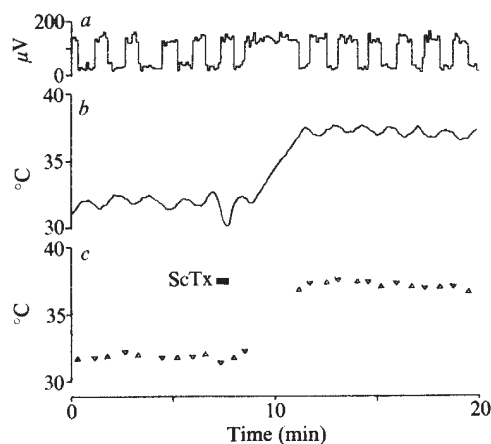


Fig. 3 Change of blocking temperature in single fibre after application of scorpion venom. a, Peak action potential amplitude (from sample and hold circuit applied to signal as in Fig. 2 upper traces). b, Temperature of nerve in test chamber. c, Temperatures at which the single fibre blocks (Δ) and unblocks (∇). At ScTx the bar indicates the time during which the scorpion venom ($2.5 \times 10^{-5} \text{ g ml}^{-1}$) was applied and perfusion by Ringer solution was suspended.

When a region of demyelination was positioned within the chamber, single fibres could be found that blocked below 37°C. The blocking temperature could be estimated crudely by varying the temperature in small steps and recording the responses when the fibre was mainly conducting and when it was mainly blocking (for example, Fig. 2a and b). Simultaneous biphasic recording of the action potential proximal and monophasic recording distal to the chamber confirmed the site of temperature block. Brief application of scorpion venom to such a unit always resulted in an elevation of the blocking temperature (for example, Fig. 2c and d).

To measure the change in blocking temperature more accurately, and to determine the stability and time course of the change, a negative feedback circuit was devised. After each biphasic spike, a gate was opened to sample the peak amplitude from the monophasic channel. If this amplitude was above the maximum noise level, the temperature of the Ringer solution perfusing the chamber was raised slowly, whereas if it was within the noise range the temperature was lowered. By this means the temperature of the root was made to oscillate slowly about the blocking temperature (Fig. 3). After a short application of scorpion venom (during which Ringer flow and temperature control were suspended) a new, higher blocking

temperature was reached, and maintained in spite of the continuous washing with fresh Ringer solution. A further application of venom (not illustrated) raised the blocking temperature to above 40 °C and it remained elevated, although declining slowly, until the termination of the experiment 50 min later.

Taking the two types of experiment together, the effect of a brief application of scorpion venom to demyelinated single fibres was to raise their blocking temperature, by 3.6°–12.4 °C ($n = 6$). This compares with the observation that a reduction in body temperature of only 0.3°–0.7 °C is sufficient to provide temporary relief of disability resulting from the lesions of multiple sclerosis. Assuming this relief is due to the prolongation of action potential duration, then a pharmacological agent with only a fraction of the potency of scorpion venom should achieve at the very least the beneficial effects of temperature reduction in this disease.

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Does substance P mediate slow synaptic excitation within the myenteric plexus?

THE widespread distribution of substance P within neurones of the central^{1–6} and peripheral^{7–9} nervous systems has led to the suggestion that it may be an important modulator of neuronal function. Although when applied by microiontophoresis^{10–14} substance P usually excites central neurones, this excitation is rather slow and this may not be appropriate to the role of a rapidly acting neurotransmitter¹¹. In this report we show substance P to have a powerful depolarising action on the membrane of myenteric neurones and that this is brought about primarily by an inactivation of resting potassium conductance. Furthermore, in several neurones we have observed synaptically evoked depolarisations which have a time course and ionic mechanism similar to the depolarisations produced by substance P. This is of particular interest in view of the evidence that the myenteric plexus contains substance P within nerve terminals and varicosities^{8,9}.

Intracellular recordings were made from neurones within isolated ganglia of the guinea pig myenteric plexus. The ganglia, adherent to the longitudinal muscle layer, were maintained in a Krebs solution at 37 °C; the composition of the Krebs solution and the details of the recording technique have been published¹⁵. Substance P (Beckmann) and other drugs were applied to the neurones by changing the bathing Krebs solution to one which differed only in its content of the drug. In low concentrations, substance P (3–100 nM) caused a dose-related depolarisation of the soma membrane. This was of rapid onset and reversed on washing with drug-free control Krebs solution. In experiments with extracellular recording the excitation evoked by substance P was not affected by blocking transmitter release with calcium-free solutions¹⁶.

Ionophoretic application of substance P directly onto the soma membrane caused pronounced depolarisations. Effects were observed with ejected charges as low as 4 nC (40 nA, 100 ms) and neuronal sensitivity ranged from 0.02 to 1.0 mV nC⁻¹. Large substance P currents depolarised the neurones to or beyond the zero potential level. The latency of the depolarisation ranged from 1 to 10 s and the total duration from 30 to 300 s. In any single neurone, the depolarisation produced by ionophoretic application of substance P (4–500 nC) was comparable in amplitude with that caused by application of a known concentration (3–300 nM) in the bathing solution. The depolarisation caused by substance P was unaffected by hexamethonium (100–200 μ M), atropine (1–2 μ M), naloxone (1 μ M) or enkephalin (1 μ M).

The depolarisation caused by substance P was associated with an increase in neuronal input resistance (Fig. 1a, b); this was not due to rectification because it still occurred when the

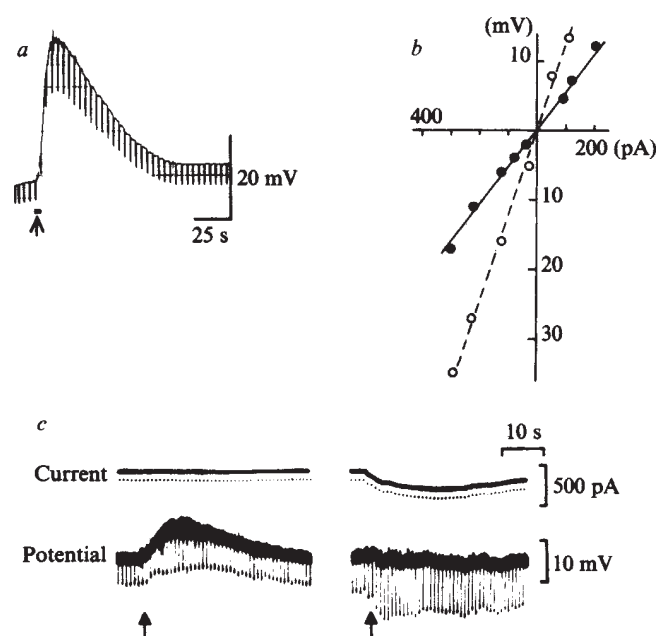


Fig. 1 Effects of substance P on single myenteric neurones. Substance P was applied by iontophoresis. The iontophoresis electrode contained substance P (2.5–10 mM) either in aqueous solution or in a sodium acetate buffer (pH 5.4) and had a tip diameter of 2–4 μ m. The tip of the electrode was placed on the soma of the impaled neurone under visual control (Nomarski optics, 500 $\times$ magnification). Ejection of currents from electrodes containing only sodium acetate buffer did not affect membrane potential (double-barrelled electrodes were used in many experiments). *a*, Constant current pulses (100 pA) were passed across the soma membrane at a frequency of 0.25 Hz. Substance P (40 nA, 5 s) was ejected at the arrow. The depolarisation caused by substance P was associated with a substantial increase in neuronal input resistance. *b*, Voltage/current relationship for a single myenteric neurone before (●) and during the depolarisation caused by the application of substance P (80 nA, 5 s) (○). Abscissa: transmembrane current amplitude (pA). Ordinate: steady-state membrane potential displacement (mV), upwards indicates depolarisation. This neurone was depolarised 17 mV by the application of substance P (not indicated on graph). *c*, Transmembrane current (upper trace) and membrane potential (lower trace) of a single myenteric neurone. At the arrows, substance P was applied to the neurone by iontophoresis (100 nA, 5 s). Left panel: the depolarisation caused by substance P was associated with an increase in neurone input resistance. Right panel: the depolarisation caused by substance P was annulled by passing a current through the recording electrode (manual voltage clamp). Substance P still caused an increase in input resistance, which is therefore not a consequence of the depolarisation. In fact, depolarisation of myenteric neurones usually causes a reduction in input resistance (not shown).

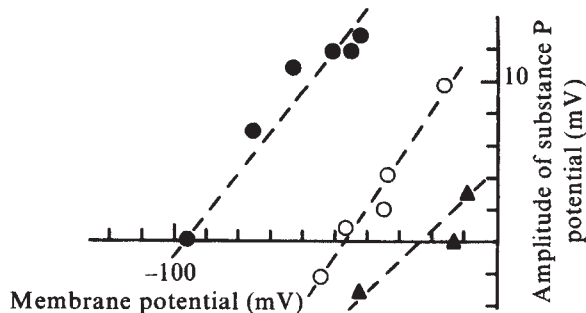


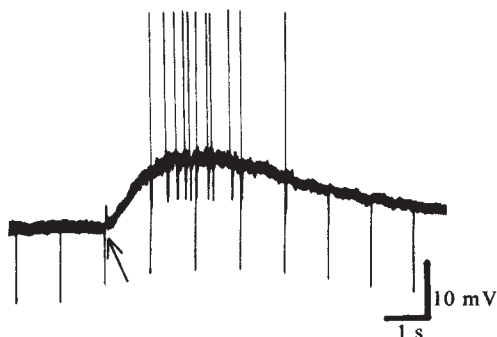
Fig. 2 Relationship between amplitude of the substance P potential (ordinate, depolarisation upwards) and the resting membrane potential at which it is elicited (abscissa). ●, Normal potassium concentration (4.7 mM); ○, 20 mM potassium; ▲, 47 mM potassium. Increasing the extracellular potassium concentration caused a shift in the substance P equilibrium potential to a more positive value.

substance P depolarisation was annulled by a manual voltage clamp method (Fig. 1c). The relationship between the amplitude of the substance P potential and the membrane potential at which it was evoked was approximately linear (Fig. 2) and the substance P potential reversed in polarity at membrane potentials of approximately -90 mV (Fig. 2). Increasing the extracellular potassium concentration caused a marked shift in the reversal potential to a less negative level (Fig. 2).

A small proportion of myenteric neurones of both types^{15,17} responded to focal stimulation (10 Hz for 1 s, pulse duration 500 μ s) of the ganglion surface with a slow depolarisation at a 100 ms–5 s latency and with a duration of 10–100 s (Fig. 3). It was abolished in calcium-free solutions (which also contained 5 mM magnesium) and was therefore judged to be synaptically evoked; however, it was unaffected by atropine (1 μ M) or hexamethonium (100 μ M). The synaptically evoked depolarisation was increased in amplitude by membrane depolarisation and reduced in amplitude by membrane hyperpolarisation; like the substance P potential it was associated with a concomitant increase in neuronal input resistance (Fig. 3).

The low concentrations of substance P which are effective and the fact that it both depolarises and increases input resistance mark substance P as a powerful modulator of neuronal

Fig. 3 Membrane potential of a single myenteric neurone. Constant current pulses (480 pA) were passed across the soma membrane at intervals of 1 s. A single focal stimulus (duration 500 μ s) was applied to the ganglion surface at a distance of 50 μ m from the soma of the impaled neurone (arrow). This evoked a slow depolarisation, associated with an increase in neurone input resistance, which reached threshold for action potential initiation. The full amplitude of the action potentials is not shown. The evoked response disappeared in a calcium-free solution (not shown). (Spikes have been retouched.)



activity. Our experiments do not allow us to conclude that substance P is an excitatory transmitter within the myenteric plexus. However, the mechanism of action of substance P and a substance released by nerve stimulation do seem to be closely similar. Experiments to find whether the synaptically evoked depolarisation can also be simulated by other endogenous constituents of myenteric plexus such as vasoactive intestinal peptide or 5-hydroxytryptamine are in progress.

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Is the quantum of transmitter release composed of subunits?

THE quantal theory of transmitter release proposed by del Castillo and Katz¹ states that the endplate potential is built up of unit potentials (quanta), each of which is the size of the spontaneously occurring miniature endplate potentials (m.e.p.s). It has recently been proposed that unit potentials and m.e.p.s are built up of 2–15 still smaller subunits^{2–4}. The basis for this proposal has been drawn in part from two observations. First, some preparations show a distinct class of small-mode m.e.p.s of amplitude typically about 1/7 that of the average m.e.p.p. Second, amplitude histograms of m.e.p.s display multiple peaks whose amplitudes sometimes appear to be integer multiples of the small-mode m.e.p.p. amplitude^{2–4}. We now present evidence suggesting that the peaks on histograms of m.e.p.p. amplitudes may arise from random variation in the data due to a limited sample size. The data do not support the proposal that m.e.p.s and unit potentials are composed of 2–15 subunits.

Experiments were carried out using frog sartorius and cutaneous pectoris neuromuscular junctions bathed in a Ringer solution containing 116 mM NaCl, 2 mM KCl, and 2 mM CaCl₂. In some experiments K⁺ was increased to 5 mM to increase the rate of m.e.p.s. The pH of the solution was held at 7.3 by 2 mM TES buffer (*N*-tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid), or phosphate buffer, and the temperature was maintained at 25°C. In an initial series of experiments m.e.p.s were recorded using standard intracellular techniques¹. Histograms of amplitudes of m.e.p.s from these experiments consistently showed peaks similar to those reported previously. In subsequent experiments we voltage clamped the endplate using standard two-microelectrode techniques⁵, and measured the areas of the miniature endplate

currents (m.e.p.cs) that give rise to the m.e.p.p. Using this method of estimating the net charge transfer during the m.e.p.p. has two advantages over conventional measurements of m.e.p.p. amplitude alone: first, it minimises errors due to noise in the recording system, and second, it prevents errors due to shifts in membrane potential.

M.e.p.cs were recorded on FM tape during the experiment and later played into a PDP-11 computer which detected each m.e.p.c. and calculated its area by numerical integration. Errors in estimates of the area due to sampling and converting the data to digital format did not exceed 2% and were usually less than 0.4%. The data were not analysed further unless two stability criteria were met. First, the mean area of successive groups of 500 m.e.p.cs had to remain constant within less than 5%. (M.e.p.cs smaller than about 1/6 the mean were usually excluded from the averages since their frequency often increased with time.) Second, the average time constant of decay of the m.e.p.cs had to remain stable within less than 5–10%. A stable time constant of decay suggests a constant membrane potential, as the time constant of decay is a function of membrane potential⁵.

For each stable set of data, histograms of m.e.p.c. areas were plotted using the moving bin method⁶ which reduces the effect of starting point and bin size on the shape of the histograms. The m.e.p.c. areas were first distributed into 300 different bins. The histograms were then constructed by plotting the number of observations in the approximately 300 overlapping sets of three consecutive bins. A moving bin analysis of this type is similar to dividing the data into 100 bins, about the same number used by previous investigators. (Similar conclusions were also obtained with conventional histogram techniques.)

Figure 1a and b presents histograms of 300 and 1,800 m.e.p.c. areas recorded from one endplate. In each histogram there are definite peaks that often appear to occur at regular intervals, just as has been reported for histograms of m.e.p.p. amplitudes^{2–4}. We also observed in some experiments small m.e.p.cs (arrow) which could give rise to the small-mode m.e.p.ps that have been reported by others^{2,3,7,8}.

Peaks on histograms like those in Fig. 1 might arise because the m.e.p.c. is composed of subunits (subunit hypothesis). A simpler explanation is that the peaks arise from random variation in the histograms due to an insufficient sample size (random variation hypothesis).

If the peaks in histograms arise from subunits, then increasing the sample size should average out some of the random variation in the data, making the nonrandom subunit phenomena more distinct. If the peaks arise instead from random variation in the data, then increasing the sample size should reduce the variation and make the peaks less distinct. Consistent with the random variation hypothesis, note in Fig. 1 that the relative prominence of the peaks diminishes as the sample size increases from 300 to 1,800.

Moreover, if the peaks in histograms arise from subunits, then the position of the peaks in histograms constructed from different subsets of the same data should all superimpose. If the peaks arise instead from random variations in the data, then the position of the peaks in different subsets of the same data should vary randomly between histograms. Figure 1c presents enlargements of corresponding segments from two histograms plotted from different subsets of 600 m.e.p.cs drawn from the data shown in Fig. 1b. Note that the peaks in these histograms usually fall in different places, arguing against the subunit hypothesis and supporting the random variation hypothesis. Note also from Fig. 1 that the spacing between peaks both within a histogram and between histograms is often quite different, as would be expected if the peaks arise from random variation.

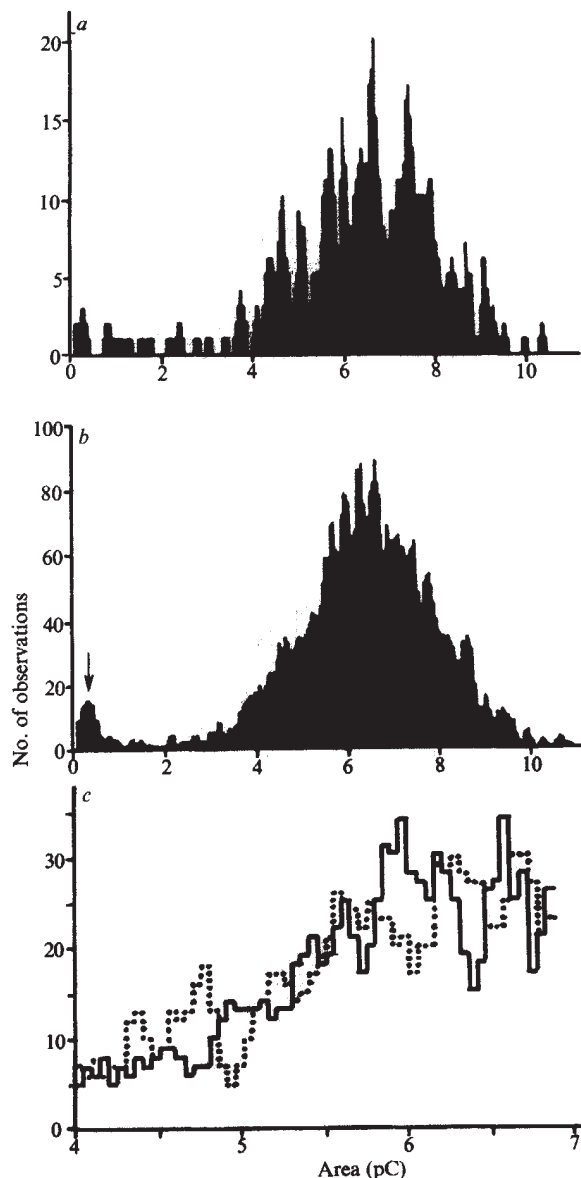
Another argument against the subunit hypothesis is that the variance (width) of the peaks in Fig. 1 does not increase with increasing m.e.p.c. areas; the variance should increase⁹ if the m.e.p.c. is composed of integral multiples of a subunit which

has variance. It seems unlikely that the subunit would have no variance.

The data from six additional experiments similar to the one shown in Fig. 1 supported the random variation hypothesis: the peaks became less prominent as the sample size was increased; the peaks of different histograms from the same set of data did not often superimpose; the spacing between peaks usually was not constant; and the variance of the peaks typically did not increase as the area increased.

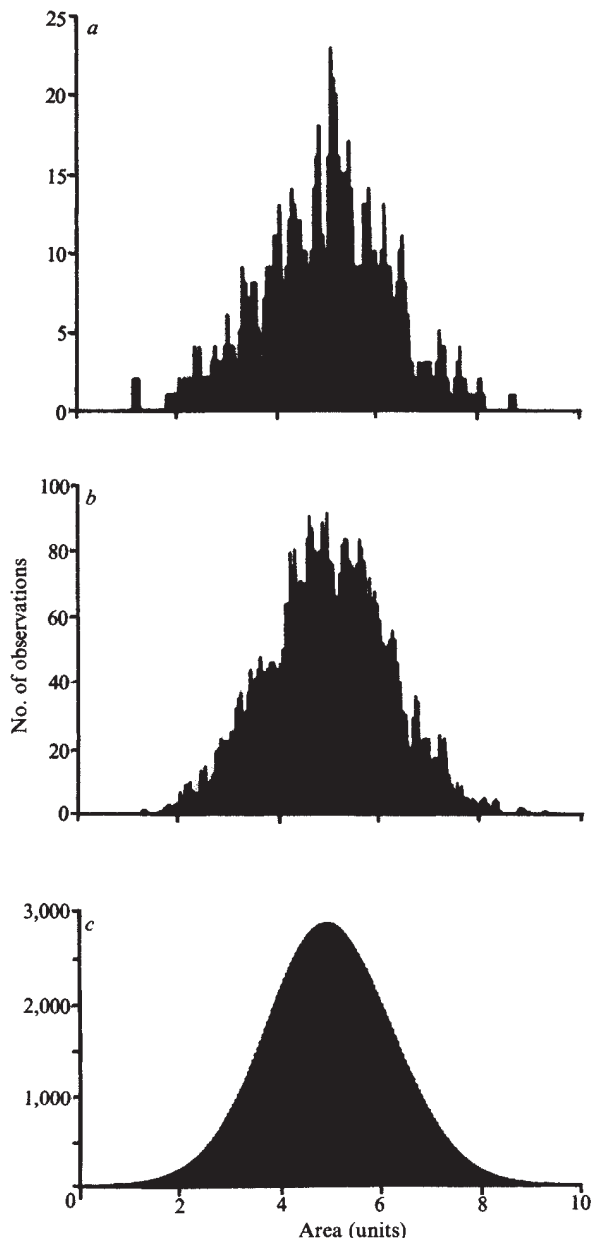
To test further whether random variation could give rise to peaks on histograms like those shown in Fig. 1, we developed a computer model of the nerve terminal. This model can

Fig. 1 Histograms of m.e.p.c. areas. A total of 1,800 m.e.p.cs were recorded from a single cell. *a*, Histogram for 300 m.e.p.cs constructed by including every sixth m.e.p.c. in the total distribution of 1,800. *b*, Histogram of all 1,800 m.e.p.cs. *c*, Enlarged views of corresponding segments from two histograms constructed from two different subsets of 600 m.e.p.cs. These subsets were selected by taking the first, fourth, seventh, ... m.e.p.cs throughout the 1,800, and the third, sixth, ninth, ... m.e.p.cs throughout the 1,800. Note that the peaks do not superimpose. Similar conclusions could be obtained if the subsets of m.e.p.cs for each histogram were selected consecutively from the 1,800 m.e.p.cs. Histograms were plotted by the moving bin method⁶ (see text). Holding potential, -90 mV; 5 mM K^+ .



generate 60,000 different m.e.p.cs, with areas distributed in a gaussian manner. Each m.e.p.c. has an equal probability of occurring, and when a m.e.p.c. is used, it is replaced with one of the same area. Figure 2 shows that while the histogram of all 60,000 m.e.p.cs does indeed describe a gaussian distribution, the histograms based on smaller numbers of m.e.p.cs drawn at random from this smooth distribution show peaks, just like those observed in the experimental data in Fig. 1. Peaks were evident, although smaller, even in histograms of 10,000 simulated m.e.p.cs. For any given set of simulated or experimental data, the number of apparent peaks could be altered at will by changing bin size. Decreasing the number of bins smoothed the distribution while increasing the number of bins increased the number of peaks.

Fig. 2 Histograms calculated using a model of the nerve terminal in which the m.e.p.c. areas are gaussian-distributed. *a*, Histogram for 300 simulated m.e.p.cs. *b*, Histogram for 1,800 simulated m.e.p.cs. *c*, Histogram of all 60,000 m.e.p.cs in the model nerve terminal. Histograms were plotted by the moving bin method⁶. Appropriate precautions and tests were made to insure that the theoretical results were not due to any systematic errors in the random number generator used to select simulated m.e.p.cs.



In conclusion, our results suggest that the peaks that we observed in histograms of m.e.p.c. areas and m.e.p.p. amplitudes most likely arise from random variation in the data due to a limited sample size. Our data do not lend support to the subunit hypothesis, but are not sufficient to exclude it. Some of the experiments that have been published by other investigators^{3,4} do seem to meet many of the criteria expected on the basis of the subunit hypothesis. Further experiments will be needed to determine whether the quantum of transmitter release is composed of 2–15 subunits. Our findings point to the need for quantitative methods to distinguish peaks in histograms arising from random variation from those that may arise from the proposed subunits.

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Ca pump driven by ATP in squid axons

If Ca^{2+} were distributed passively across the nerve membrane, the existence of a membrane potential of 50–60 mV negative with respect to the external medium would lead to an accumulation of Ca inside the cell. However, ionised Ca is normally 10^3 -fold less concentrated inside the cell than it is outside^{1–3}, a difference which is essential for the normal function of excitable preparations such as muscles and the synaptic regions of neurones. The observation that the extrusion of Ca depends in part on external Na led to the proposal that the energy for Ca extrusion may be derived from the transmembrane Na electrochemical gradient^{4,5}. More recently, it has been found that cellular metabolic energy in the form of ATP interacts with the Ca transporting system^{6,7}. Nevertheless, its role as an energy source for Ca transport is still something of a puzzle. At present, it is not clear whether ATP is consumed when Ca is transported across the axolemma or whether it only interacts with the Ca-transporting system without itself being hydrolysed. On the assumption that ATP might energise Ca transport, it should be possible to demonstrate an ATP-driven net Ca extrusion in the absence of other energy sources such as those derived from the normally existing ionic gradients across the membrane. We report here experimental evidence for a direct involvement of ATP as an energy source for Ca transport in squid axons.

Our experiments were carried out on giant axons of the squid *Dorytheutis plei*. This preparation is suitable for controlling the intracellular concentration of low molecular weight solutes using the dialysis technique⁸. We have designed a method to measure both unidirectional Ca fluxes in a single squid axon, thus avoiding the variability in flux measurements from axon to axon. The experimental procedure consists of determining the magnitude of the Ca influx from a segment of an axon in which the external radioactive medium is restricted only to the dialysed portion of the axon. The subsequent removal of the external radioactive solution followed by dialysis with a

medium containing ^{45}Ca enables the Ca efflux from the same segment of the axon to be determined. To eliminate all the ionic gradients that normally exist across the membrane, axons were perfused internally and externally with essentially the same solution. The main composition of the standard medium was (mM): K^+ , 10; Na^+ , 90; Mg^{2+} , 10; Tris^+ , 400; pH, 7.2. $[\text{Ca}^{2+}]_i$ was set at $0.45 \mu\text{M}$ with the use of EGTA (2 mM). The

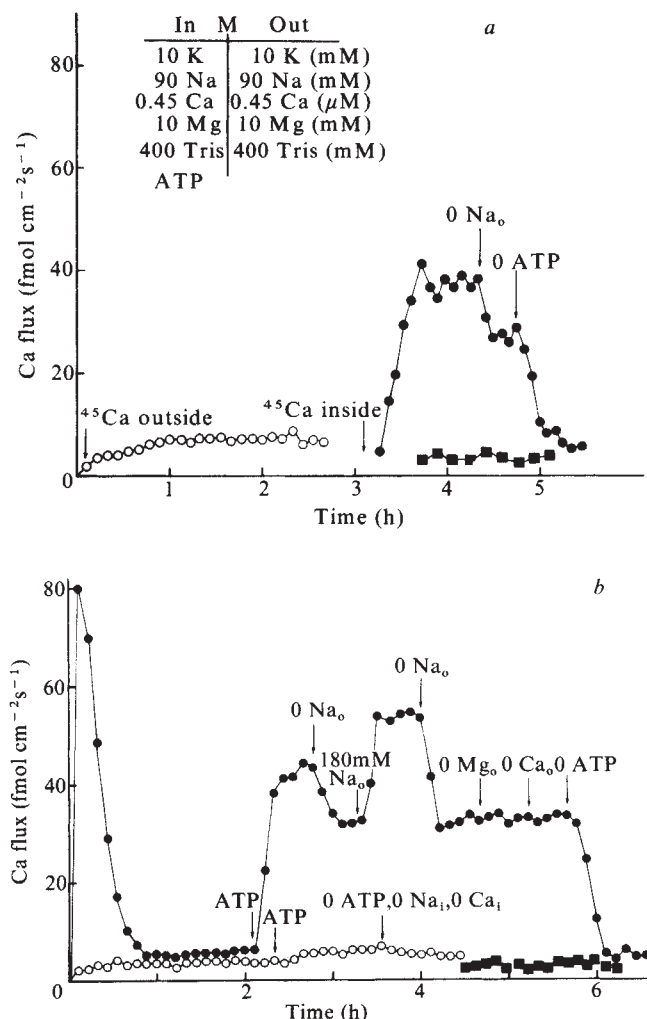


Fig. 1 *a*, ^{45}Ca influx ($\circ$) and efflux ($\bullet$) from internally dialysed squid axons, and the effect of ATP on the magnitude of both unidirectional Ca fluxes in a single dialysed squid axon. Zero ionic gradients were obtained using the standard medium on either side of the membrane; ionic concentrations are given in the text. The fibre was dialysed with the standard medium containing 1 mM Mg ATP on the inside. Cyanide and oligomycin were also included in the internal medium. For Ca influx measurement, the ^{45}Ca -containing solution was added to the fluid superfusing the axon and the internal perfusate collected at 7-min intervals. The ordinate represents Ca flux in $\text{fmol cm}^{-2} \text{s}^{-1}$, the abscissa, time in h. After measuring ^{45}Ca influx, the radioactive external solution was removed and the experimental chamber washed carefully. During this period (30 min), the axon was continuously dialysed to remove the isotope accumulated during influx measurement. The efflux of ^{45}Ca was measured by perfusing the dialysis capillary with a radioactive standard medium. The magnitude of the CaEGTA leakage ($\blacksquare$) refers to a total [EGTA] of 1.5 mM. The external temperature was 19°C . Axon diameter $475 \mu\text{m}$. *b*, The effect of Na_o , Ca_o and Mg_o on the ATP-dependent Ca efflux. Before starting the dialysis the axons were incubated for 1 h in artificial seawater containing 1 mM NaCN to lower their ATP content. Ca influx and Ca efflux were measured in two separate axons. The arrows indicate changes in the internal and external experimental conditions. Nominally zero calcium solutions were obtained by adding 2 mM free EGTA to the standard medium.

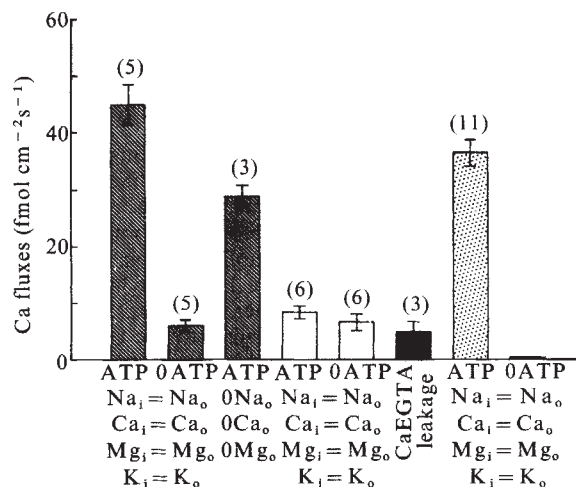
absolute magnitudes of the unidirectional Ca fluxes were evaluated by measuring the leakage of CaEGTA through the membrane using ^{14}C -EGTA.

In the axon used in Fig. 1*a*, the magnitudes of both unidirectional Ca fluxes were investigated in the absence of ionic gradients across the membrane. A steady-state Ca influx level of about $6 \text{ fmol cm}^{-2} \text{s}^{-1}$ (f/CS) is obtained in the presence of ATP. This value is not significantly different from that obtained for the leakage of CaEGTA ($4 \text{ fmol cm}^{-2} \text{s}^{-1}$) measured in the same axon. A quite different result is found for the Ca efflux. An activation up to $40 \text{ fmol cm}^{-2} \text{s}^{-1}$ is observed in the presence of internal ATP. Replacement of all the Na_o by Tris reduces the efflux by 36%, and removal of ATP from the dialysis fluid reduces it to near the leakage of CaEGTA. Similar experiments to that already described (see Fig. 2) clearly show that ATP induces an asymmetric activation of the Ca fluxes in the absence of an electrochemical Na gradient. This behaviour is to be expected if metabolic energy is directly used in the active extrusion of Ca^{2+} .

Figure 1*b* shows the results of two experiments which were designed to explore in more detail the ionic requirement of the ATP-dependent net Ca efflux. It can be observed that at zero ionic gradients across the membrane and in the absence of ATP, influx and efflux are not significantly different in magnitude. ATP increases the Ca efflux to a steady value of about $42 \text{ fmol cm}^{-2} \text{s}^{-1}$. Clearly, a component of this ATP-activated Ca efflux is dependent on Na_o , as it is partially reduced by the removal of Na_o . Subsequent exposure to 180 mM Na_o increases the Ca efflux to a value higher than that found at 90 mM Na_o . Interestingly, the component of the ATP-dependent Ca efflux that persists in the absence of Na_o is insensitive to the removal of Mg_o and Ca_o . This component is completely dependent on ATP, as its removal from the internal medium reduces the Ca efflux to near the value of the CaEGTA leakage.

Figure 2 summarises the results of several experiments in which the absolute magnitudes of the Ca fluxes were measured

Fig. 2 Comparison of Ca efflux, Ca influx and net Ca flux in the presence and absence of ATP. Ordinate, Ca flux in $\text{fmol cm}^{-2} \text{s}^{-1}$; abscissa, experimental conditions during steady-state Ca flux measurements. The main composition of the standard medium is given in the text. Cyanide and oligomycin were included in the dialysis medium to eliminate mitochondrial Ca uptake. Each bar represents the mean of the number of experiments shown in parentheses; error bars indicate $\pm \text{s.e.m.}$ The average temperature was 18.5°C . The first two bars show the effect of ATP on the Ca efflux in the absence of ionic gradients across the membrane. The third bar represents the magnitude of the Na_o , Ca_o and Mg_o independent, ATP-dependent Ca efflux. Note that Ca influx during the experiment is not significantly different from the leakage of CaEGTA. The last bar represents the net Ca efflux in the presence of ATP and in the absence of ionic gradients; net Ca flux was calculated by subtracting the absolute values of Ca efflux and influx.



in the presence and absence of ATP. The following points emerge: in conditions of no ionic gradients across the membrane no net Ca flux exists in the absence of internal ATP. In fact, both Ca influx and efflux are similar in magnitude to the leakage of CaEGTA; ATP promotes a net outward movement of calcium in the absence of ionic gradients across the membrane. A fraction of this ATP-dependent Ca extrusion depends on external Na. Particularly interesting is the finding that a large fraction of the ATP-dependent Ca efflux is apparently not coupled to any external ion. The existence of an uncoupled Ca pump in squid axons has been suggested by Baker and McNaughton⁹ to account for the residual Ca efflux observed in unpoisoned axons in the absence of external Na and Ca.

In conclusion, these data constitute the first direct demonstration of an ATP-driven Ca pump in squid axons. Two further indirect findings favour an ATP-requiring net Ca efflux: first, only hydrolysable analogues of ATP (2 deoxy-ATP and AMP-CP) are able to activate Ca efflux; the nonhydrolysable analogue AMP-PCP competes with ATP for the internal activating site¹⁰, and second, we have recently found an ouabain insensitive Ca-activated ATPase in purified membrane fractions of lobster nerve fibres (Proverbio and R.D., unpublished results). Although further work is necessary to elucidate the physiological relevance of this finding, a potentially important role of an ATP-dependent Ca efflux in the maintenance of the physiological $[Ca^{2+}]_i$ is suggested by the recent observation that at physiological levels of $[Ca^{2+}]_i$ (0.02–0.06 μ M) (ref. 3) no Na-dependent Ca efflux is observed in the absence of ATP.

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Immunochemical identification of renin in rat brain and distinction from acid proteases

THE direct action of angiotensin II on the central nervous system is well recognised^{1–4}. There is evidence for the presence in the brain of angiotensinogen⁵, angiotensins^{6–9}, and angiotensin I converting enzyme^{10–12}. These findings strongly suggest the presence of a brain renin–angiotensin system which may function independently from the somatic system. Renin-like activity in the brain has also been reported^{8,13,14}. However, the acid pH optimum of the angiotensin I-generating activity in the brain^{13,14} led to the suggestion that this renin-like activity might be due to a nonspecific action of acid proteases released during homogenisation and that intrinsic brain renin might not exist¹⁴. Since this view has a profound implication on the origin and control of brain renin activity, we have sought to obtain an unequivocal answer as to whether the observed renin-like activity is indeed due to renin intrinsic to the brain. Using an affinity column capable of separating renin from general acid proteases, and by making use of specific antibodies to renin elicited by pure pig renin¹⁵. We demonstrate here the presence in rat brain of renin which is clearly distinct from acid protease.

Antisera against pig renin was prepared by a series of intradermal injections of pure pig renin conjugated to pure

tetanus toxoid with Freund's complete adjuvant¹⁶. This antiserum had been shown to neutralise 50% of rat brain renin activity at 1:3000 dilution. It was used here at 1:30 dilution to ensure the complete reaction of antibody and renin in brief incubations.

Affinity gel capable of preferentially binding acid proteases without appreciable affinity to renin was prepared by coupling 800 mg of bovine α -casein (Worthington) to 50 ml of agarose gel (Sephacrose 4B, Pharmacia) previously activated by cyanogen bromide by the method of March *et al.*¹⁷. Renin activity was determined by the radioimmunoassay of angiotensin I¹⁸ generated from renin-free rat plasma obtained 40 h after bilateral nephrectomy. Acid protease activity to cleave denatured ¹⁴C-labelled haemoglobin was measured by the method of Williams and Lin¹⁹.

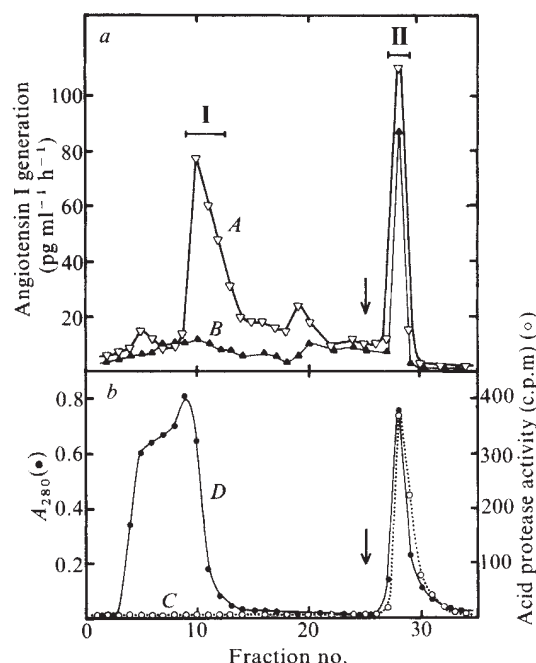


Fig. 1 Separation of renin from acid protease in brain extract by a column of casein–Sephacrose gel. The extract (16 ml) of the brain of a nephrectomised rat dialysed against 2 l of 0.05 M acetate buffer, pH 3.5 for 4 h, centrifuged at 10,000 g for 20 min to remove precipitates, was applied to a casein–Sephacrose column (1.6 × 4.0 cm) previously equilibrated with the same buffer. After wash with 72 ml of the same buffer, 0.1 M Tris–acetate buffer, pH 8.6, containing 1 M NaCl was applied at the point indicated by the arrow. The angiotensin I-generating activity (curve A) was determined by the radioimmunoassay of angiotensin I produced by incubating 100 μ l of each fraction with 25 μ l of plasma renin substrate obtained from nephrectomised rats at 37 °C for 24 h in 0.20 M maleate buffer, pH 6.0, containing 6 mM EDTA and 2 mM phenylmethanesulphonylfluoride. Effect of renin specific antisera on the angiotensin I-generating activity (curve B) was determined similarly after incubation of 100 μ l aliquots of the fractions with 10 μ l of rabbit anti-pig renin antiserum (1:30 dilution) at 4 °C for 15 h at pH 8. Corrections were made for the renin activity present in the rabbit antisera. The acid protease activity (curve C) was determined by the method of Williams and Lin¹⁹ by incubating 50- μ l aliquots of the fractions with 250 μ l of 0.4 M Na-acetate buffer, pH 3.5, containing 50 μ g of ¹⁴C-labelled haemoglobin at 37 °C for 2 h. Protein concentration was estimated spectrophotometrically as absorbance at 280 nm (curve D). The following method was used to prepare the antiserum. Pure pig renin (500 μ g) was conjugated to 250 μ g of pure tetanus toxoid (gift of Dr John P. Robinson) by using 10 μ g of water-soluble carbodiimide in 1.5 ml of water by overnight reaction at room temperature, treated with 0.5 M hydroxylamine for 5 h then dialysed exhaustively. This conjugate (100 μ g in renin) was injected intradermally to a rabbit in multiple sites followed by 4 weekly boosters of 50 μ g each. The antiserum was collected 2 weeks after the last booster.

Adult male Wistar rats, bilaterally nephrectomised 40 h before killing were exhaustively bled through the femoral artery under pentobarbital anaesthesia and the brain was perfused with 80 ml of saline through carotid arteries. The whole brain weighing approximately 8 g was homogenised in 40 ml of 0.01 M pyrophosphate buffer, pH 6.5, containing 0.1 M NaCl in a homogeniser (VirTis 60) at 10,000 r.p.m. for 5 min. The resulting homogenate was centrifuged at 100,000g for 45 min in the cold and the supernatant was retained as rat brain extract.

The angiotensin I generating activity of the brain extract was separated into two major fractions, peak I and II (curve A in Fig. 1a). The activity under peak I showed very weak affinity to the gel and was completely neutralised by antirenin antiserum at 1:30 dilution (curve B in Fig. 1a). Preimmune rabbit sera used as control did not have any effect. The peak I fractions lacked the general protease activity to degrade haemoglobin at pH 3.5 (curve C in Fig. 1b) and at pH 5.4 (not shown). On the other hand the angiotensin I-generating activity of peak II fractions was insensitive to the antirenin antiserum (curve B) but possessed strong proteolytic activity at pH 3.5 (curve C, Fig. 1b) and at pH 5.4. The slight decrease in the angiotensin I-generating activity caused by antirenin antibodies may be due to the inhibition of a small amount of renin contaminating peak II fractions.

The angiotensin I-generating activity eluted under peak I fractions showed a pH optimum in the neutral pH region between 6.0 and 7.5 as shown by curve A in Fig. 2. This activity was completely neutralised by antirenin antisera over the entire pH range studied (curve B, Fig. 2). The angiotensin I-generating activity eluted under peak II of Fig. 1a had a pH optimum of 4.0 and showed very little activity above pH 6.0. Renin concentration of the rat brain was estimated to be ≥ 250 pg angiotensin I generated per g wet weight per h based on the total activity eluted under peak I. The plasma renin concentration of the nephrectomised rat was equivalent to < 40 pg angiotensin I generated per ml per h. Thus, renin concentration in the brain is considerably higher than the plasma level.

The casein-Sepharose affinity column was constructed for renin purification making use of the different affinities of the acid protease and renin to casein. This new affinity column makes it possible to separate renin activity in rat brain into two components. It is clear that the fraction I component is a true renin identical with the renal renin or a closely related enzyme discrete from a cathepsin-like acid protease as judged by criteria including sensitivity to specific antirenin antibody, lack of general proteolytic activity, a neutral pH optimum and unique behaviour on the casein-Sepharose column. The fact that the brain of nephrectomised and exsanguinated rats exhaustively perfused with saline shows a renin activity level much higher than the renin level of the nephrectomised plasma excludes the possibility of contamination of the brain by plasma renin of renal origin.

The fraction II component of the angiotensin I-generating activity which is optimally active at an acid pH, insensitive to antirenin antibody and possessing general proteolytic activity, seems to be a protease(s). The acid pH optimum of the angiotensin I generating activity of brain extracts observed previously is apparently due to the nonspecific action of this type of enzymes as pointed out by Day and Reid^{4,14}. The angiotensin I generating activity of this enzyme seems to be dependent on assay conditions. In brain homogenates incubated at slightly acid pH this component may become the predominant angiotensin I-generating species overshadowing the true renin fraction^{13,14}. However, its role in the intact brain in physiological conditions is not yet known. It is possible that in normal physiological conditions the protease may not express its activity due to compartmentalisation in organelles such as lysosomes.

Taken together with evidence for the presence of other components of the renin angiotensin system in the brain, the demonstration of specific renin suggests the existence of an independent brain renin-angiotensin system. Thus, it is highly

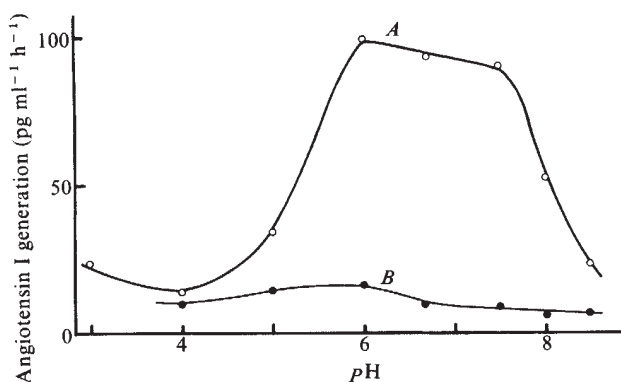


Fig. 2 The pH profile of the angiotensin I-generating activity of fraction I of rat brain extract separated by the casein-Sepharose column. Pooled fraction I indicated by solid bar in Fig. 1 was concentrated 10-fold by pressure filtration on an Amicon PM-10 filter. Aliquots (50 μ l) of the concentrate were incubated at 37°C for 24 h with 25 μ l of the plasma substrate obtained from nephrectomised rats, 10 μ l of 0.2 M EDTA, 5 μ l of 0.15 M phenylmethanesulphonylfluoride and 200 μ l of Na-citrate (0.25 M)-phosphate (0.25 M) adjusted to appropriate pH. Angiotensin I generated was determined by radioimmunoassay¹⁸. Curve A was obtained without preincubation of fraction I with the antirenin antiserum and curve B after incubating fraction I with the antirenin antiserum prior to assay as described in Fig. 1.

likely that the formation of brain angiotensin II, which has unique functions such as dipsogenesis²⁰, release of antidiuretic hormone²¹, elevation of blood pressure by a neuronal mechanism^{1,2,23} and possible neurotransmitter function⁶, is under the control of the endogenous renin in the brain. The fact that renin substrate injected into the brain causes dipsogenesis²³ can be taken as a strong indication for the presence of locally available renin functional at physiological pH.

The rat brain renin described here could well be identical to the enzymes reported in abstract form^{24,25} since submission of this manuscript.

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Cholesterol oxidase as a probe for studying membrane organisation

CHOLESTEROL is a major constituent of the phospholipid bilayer of many biological membranes. The ratio of cholesterol to phospholipid seems to be an important determinant of the physical and biological properties of membranes¹. Although several model systems now provide a molecular basis for understanding preferential interaction of cholesterol with different phospholipids^{2,3}, the distribution and location of cholesterol in lipid bilayers has not been clearly established. A recent study⁴ demonstrated marked differential susceptibility to oxidation by cholesterol oxidase (EC 1.1.3.6) of cholesterol in the inner and outer layers of the erythrocyte membrane. Another series of experiments revealed that cholesterol in a virus membrane was accessible to oxidation by cholesterol oxidase only when external phospholipid headgroups were removed by phospholipase C (ref. 5). We report here our studies on the usefulness of the enzymatic action of cholesterol oxidase to probe the membrane bilayer distribution of cholesterol and its differential interaction with choline-containing and amino-containing phospholipids. To this end, enzymatic oxidation of cholesterol was measured in mixed lipid vesicles composed of various phospholipids and cholesterol. The possible application of this technique to biological membranes was also investigated using the membranes of human erythrocytes and vesicular stomatitis (VS) virus.

Table 1 demonstrates that the cholesterol present in membranes of intact VS virus and intact human erythrocytes was essentially inaccessible to oxidation by cholesterol oxidase compared with 100% oxidation after disruption of each membrane with 0.3% sodium taurodeoxycholate. Trypsin digestion of external glycoprotein spikes of otherwise intact VS virus did not render viral membrane cholesterol accessible to cholesterol oxidase. Moreover, hydrolysis of VS virus membrane glycerophospholipids to lysophospholipids and fatty acids by bee venom phospholipase A₂ did not expose the cholesterol to oxidation by cholesterol oxidase. In sharp contrast, virtually all the membrane cholesterol could be oxidised by cholesterol oxidase when otherwise intact VS virus and erythrocytes were exposed to phospholipase C at 37 °C for four hours. Previous studies⁵ had shown that in optimal conditions phospholipase C can liberate no more than 55% of the phospholipid headgroups from VS virus, corresponding to the phospholipids in the outer monolayer of the membrane. Table 1 also shows that the amount of cholesterol accessible to cholesterol oxidase is a function of the number of phospholipid headgroups hydrolysed by phospholipase C. Cholesterol in resealed erythrocyte ghosts was also inaccessible to cholesterol oxidase unless they were previously or simultaneously exposed to phospholipase C.

Similar inaccessibility to cholesterol oxidase of membrane cholesterol in intact erythrocytes and resealed ghosts was previously reported by Gottlieb⁴, who also observed that cholesterol oxidase could oxidise most of the membrane cholesterol of 'leaky' erythrocyte ghosts, resealed ghosts with enzyme trapped inside, and 'inside-out' vesicles prepared from erythrocyte membrane. These remarkable differential effects of cholesterol oxidase acting at the two surfaces of the erythrocyte membrane might be due to the well characterised phospholipid asymmetry of the two layers of the erythrocyte membrane, whereby most of the aminophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), are present in the inner layer, compared with the predominance of the cholinephospholipids, phosphatidylcholine (PC) and sphingomyelin (SPM), in the outer layer (for review see ref. 6). This phospholipid asymmetry could explain the accessibility of inner membrane cholesterol to cholesterol oxidase, particularly in

view of the finding that cholesterol interacts with phospholipids with a preferentially greater degree of intensity in the rank order of SPM > PC > PE (refs 7, 8). Similar asymmetry in phospholipid distribution has been noted in the membrane of vesicular stomatitis virus⁹.

These observations led us to hypothesise that the predominance of cholinephospholipids with bulky headgroups in the outer monolayer of both erythrocyte and VS virus membranes provided steric hindrance to cholesterol oxidase, whereas aminophospholipids did not protect the hydroxyl group of cholesterol in the inner membrane layer. As a test of this hypothesis, unilamellar lipid vesicles were prepared by ultrasonication¹⁰ from different phospholipids, singly or in combination, but with a constant proportion of 33% cholesterol. Unfortunately, it is not possible to generate competent vesicles composed only of PE and cholesterol. All other lipid vesicles were found to be homogeneous and intact as determined by the procedures described elsewhere¹⁰.

Table 2 shows that negligible cholesterol was oxidised in intact vesicles containing SPM or PC as the only phospholipid. Phospholipase C removal of headgroups from these choline phospholipids resulted in virtually complete accessibility of cholesterol to cholesterol oxidase. A limited but significant degree of accessibility to cholesterol oxidase was exhibited by cholesterol present in intact mixed vesicles containing 33% PE and 33% PC. In sharp contrast, virtually all the cholesterol was oxidised after exposure to cholesterol oxidase of intact vesicles in which PS was the only phospholipid. One can rule out negative charge as the primary factor in promoting accessibility of cholesterol in PS vesicles because the presence of up to 20 mol % phosphatidic acid (+80% PC) resulted in only limited enzyme accessibility to cholesterol, possibly due to a partial decrease in the number of choline headgroups.

The above observations suggest that cholesterol in association with a monolayer of aminophospholipids is available to

Table 1 Accessibility of cholesterol in intact, disrupted or enzyme-treated membranes of VS virus and human erythrocytes to cholesterol oxidase

Membrane	Treatment	% Cholesterol oxidised
VS Virus	None (intact)	3
	0.3% Deoxycholate*	100
	Phospholipase C (4 h)	98
	Phospholipase C (4 min)	43
	Phospholipase C (1.5 min)	24
	Phospholipase A ₂ (4 h)†	2
Erythrocyte	Trypsin‡	2
	None (intact)	2
	0.3% Deoxycholate*	100
	Phospholipase C (4 h)	99
Erythrocyte resealed ghosts	None (intact)	2
	Phospholipase C	99

Purified VS virus (0.1 mg ml⁻¹) or washed human erythrocytes and ghosts (1 mg ml⁻¹), untreated or treated in various ways were incubated at 37 °C for 4 h with cholesterol oxidase (0.025 units per ml). Oxidation of cholesterol to cholest-4-en-3-one was measured as described by Moore *et al.*⁵. Values represent the mean of three determinations. Incubation for 9 h did not increase the degree of oxidation. Incubation of intact VS virus, erythrocytes or ghosts with phospholipase C (0.2 units per ml) at 37 °C for 4 h resulted in ~55% hydrolysis of phospholipid headgroups⁵. Incubation of VS virus with phospholipase C for 4 min or 1.5 min resulted in phospholipid hydrolysis of 10% or 5%, respectively.

* Membranes completely disrupted with 0.3% taurodeoxycholate.

† 50–60% hydrolysis of phospholipids to lysophospholipids and fatty acids, none of which were liberated from virions.

‡ Glycoprotein spikes completely removed as determined by polyacrylamide gel electrophoresis.

Table 2 Comparative accessibility to cholesterol oxidase of cholesterol in mixed phospholipid vesicles

Phospholipid vesicles with 33% cholesterol	% Cholesterol oxidised by	
	Cholesterol oxidase alone	Cholesterol oxidase + phospholipase C
SPM, 67%	2	98
PC, 67%	3	97
PC, 33% + PE, 33%	9	—
PS, 67%	98	—
PC, 63.5% + PA, 3.5%	10	—
PC, 53.5% + PA, 13.5%	21	—

Unilamellar lipid vesicles were prepared by ultrasonication of 33% cholesterol mixed with 67% phospholipids in various proportions of beef brain sphingomyelin (SPM), egg phosphatidylcholine (PC), phosphatidylethanolamine (PE), beef brain phosphatidylserine (PS) and/or phosphatidic acid (PA)¹⁰. Preparations of 1 μ mol per ml of lipid vesicles were incubated with cholesterol oxidase alone (0.025 units per ml) or with cholesterol oxidase + phospholipase C (0.2 units per ml) for 4 h at 37°C. Oxidation of vesicle cholesterol to cholest-4-en-3-one was measured as described by Moore *et al.*⁵. As a positive control, an aliquot of each preparation of lipid vesicles was disrupted with 0.3% taurodeoxycholate which invariably resulted in 100% oxidation of the cholesterol by cholesterol oxidase as determined by absorption at 235 nm in heptane⁵.

cholesterol oxidase, whereas cholesterol in association with cholinephospholipids is not. An obvious difference between these two groups of phospholipids is headgroup size. A recent study estimated the size of the polar headgroups in SPM, PC and PE bilayers to be 20 Å², 22 Å² and 16 Å², respectively¹¹. Smaller headgroups may produce the tighter packing of acyl chains observed in PE and PS compared to PC bilayers^{12,13}, and it explains the higher transition temperature of PE and PS (refs 12, 14) than that of PC (ref. 12) or SPM (ref. 15) with identical fatty acyl chains. These effects on other regions of the phospholipid molecule in conjunction with the smaller headgroups may produce a more favourable substrate for cholesterol oxidase, or may cause the β -OH group of cholesterol to be more exposed to the aqueous environment in the amino-phospholipid-cholesterol monolayer. In addition, these properties may explain the preference of cholesterol for interaction with phospholipids in the order SPM > PC > PE (refs 7, 8). (The differences between SPM and PC can be explained mainly by differences in their interface region¹⁶.)

An explanation is also required for the means by which cholesterol oxidase, by reacting with only one membrane layer, can oxidise virtually all the cholesterol (representing 30–40% of the total lipid) in the membranes of VS virus and erythrocytes treated with phospholipase C and in untreated PS/cholesterol vesicles and inner erythrocyte membranes⁴. Previous experiments revealed that phospholipase C does not make the VS virus membrane more penetrable, as judged by failure of lactoperoxidase-catalysed iodination to label internal proteins⁵. We suggest two possible explanations for virtually 100% enzymatic oxidation of cholesterol in VS virus, erythrocyte and PS vesicle membranes: first, all the cholesterol is present in the outer layer and the enzyme penetrates after reaction with the inner erythrocyte layer; and second, cholesterol in either membrane layer flips rapidly (<2.5 min in VS virus, unpublished data) across to the opposite membrane layer. The second explanation seems far more plausible, particularly when considered in the light of recent data^{17,18}. Therefore, cholesterol oxidase would seem to provide a useful probe for studying the relative association and interaction of cholesterol with phospholipids in biological membranes. This is particularly important in view of the phospholipid asymmetry seen in many biological membranes⁶ and may be related to different functional properties of each membrane layer.

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Pathogenicity of conventional and debilitated *Escherichia coli* K12

THE development of 'disabled' strains of *Escherichia coli* for recombinant DNA work has so far concentrated on abolishing their ability to survive outside the test tube and to persist in the intestinal tract of man and animals. However, these changes make it impossible to test for any possible changes in pathogenicity in these strains caused by inserted foreign DNA by simple oral inoculation. Oral inoculation also does not provide any information on their relative pathogenicity if accidentally introduced by any other route. We have therefore investigated the ability of *E. coli* K12 DO-11 and of the 'disabled' *E. coli* χ 1776 to colonise and kill susceptible mice when introduced by various parenteral routes. We show that at high concentrations both of these *E. coli* strains, which do not colonise the intestines of normal (non-germ-free) mice, can survive and prove lethal when injected intravenously, intraperitoneally or intracranially.

Table 1 Survival of mice

Route of inoculation	DO-11		χ 1776	
	Expt 1	Expt 2	(2 $\times$ 10 ⁸)*	(1.5 $\times$ 10 ⁹)*
	(6 $\times$ 10 ⁸)* No. dead/total	(2 $\times$ 10 ⁹)* No. dead/total	(2 $\times$ 10 ⁸)* No. dead/total	(1.5 $\times$ 10 ⁹)* No. dead/total
Intraperitoneal	0/10	—	—	4/10
Intraperitoneal + mucin	3/10	3/10	1/15	4/10
Mucin alone	0/5	—	—	0/5
Intravenous	4/10	3/10	—	3/10
Intravenous (boiled)	—	0/5	—	0/5
Intracranial†	10/10	—	—	10/10
Intracranial (boiled)†	—	3/5	—	1/5

* No. of viable cells inoculated.

† All dead within 48 h of inoculation.

Table 2 Recovery of bacteria from inoculated mice

	Liver	Strain DO-11 Spleen	Lung	Liver	Strain χ 1776 Spleen
Intraperitoneal					
Day 2	—	—	—	4/4 (10^3 – 10^4)	4/4 (10^3 – 10^4)
Day 5	2/5* (10^3)	3/5 (10^3)	2/5 (10^2)	0/6	0/6
Intraperitoneal & mucin					
Day 1	3/3 (10^3 – 10^4)	3/3 (10^3 – 10^4)	1/1 10^3	2/2 (10^3 – 10^4)	2/2 (10^3 – 10^4)
Day 2	4/4 (10^2 – 10^3)	4/4 (10^3 – 10^4)	2/2 (10^1 – 10^2)	2/2 (10^3 – 10^4)	2/2 (10^4)
Day 5	4/5 ($10^{0.9}$ – 10^2)	4/5 (10^2)	2/4 (0 – $10^{0.2}$)	0/6	0/6
Intravenous					
Day 1	3/3 (10^3)	3/3 (10^3)	3/3 (10^3)	1/1 (10^4)	1/1 (10^2)
Day 2	1/1 (10^1)	1/1 (10^2)	1/1 (10^1)	2/2 (10^2)	2/2 (10^2)
Day 5	0/7	0/7	0/4	0/7	0/7

* Number positive-culture/number tested (concentration of viable bacteria per g tissue).

When given orally the laboratory derivative *E. coli* K12 fails to colonise the intestines of mice which have a complete intestinal flora (ref. 1 and S.B.L., J. Davis, B. Marshall, M. Richter and A. Onderdonk, unpublished data), but will colonise germ-free mice. The debilitated bile-sensitive *E. coli* χ 1776, constructed by R. Curtiss III and colleagues² did not colonise the intestines of germ-free mice even at an inoculum of 10^9 – 10^{10} bacteria introduced by gastric intubation (S.B.L. *et al.*, unpublished data). The killing capacity and survival in mice of conventional and debilitated *E. coli* K12 introduced by various parenteral routes were assessed with *E. coli* DO-11, a derivative of CSH-2, into which nalidixic acid resistance had been introduced³, and *E. coli* χ 1776, a multiply-auxotrophic, bile-sensitive, diaminopimelic-acid requiring mutant of *E. coli* K12 (ref. 2). The bacteria were grown in L broth⁴ supplemented with required nutrients to a density of 5×10^8 cells ml^{-1} collected by centrifugation, and resuspended at a concentration of 10^{10} cells ml^{-1} in buffered saline. Samples were injected by various routes into male CD-1 mice. Inoculum doses of 10^8 to 10^9 bacteria were injected into each animal since preliminary experiments indicated that these levels were near the LD_{50} for intravenous injection.

Both χ 1776 and DO-11 caused death by all routes (Table 1). The mortality was 30% to 40% by intravenous (i.v.) and intraperitoneal (i.p.) routes. The mean period between challenge and death was 36–48 h. There seemed to be no difference in the LD_{50} of the two bacterial strains. To exclude endotoxin-induced death, 2×10^9 cells of strain DO-11 were boiled and injected i.v.; all animals survived. The most lethal route was intracranial (i.c.): animals challenged in this manner were all dead within 2 d. However, heat-inactivated organisms injected i.c. also caused some deaths, so it is probable that endotoxin was responsible in part for the high mortality by this route. Mice surviving for 5 d seemed to have recovered from the bacterial challenge and were no longer in danger of dying.

The second stage of the study explored whether viable bacteria could be recovered from the liver, spleen and lungs of inoculated animals. Organs were removed sterily from mice within hours of death on days 1 and 2 and tested for presence of the inoculated bacteria. Animals surviving 5 d were killed and their organs were also tested for viable bacteria. The liver, spleen and lungs were removed and pieces were minced and homogenised in physiological saline. After sedimentation of organ tissues, samples of the supernatants were plated for quantitative studies on MacConkey agar supplemented with nalidixic acid ($30 \mu\text{g ml}^{-1}$) for DO-11 and on L broth agar supplemented with diaminopimelic acid ($100 \mu\text{g ml}^{-1}$) thymidine ($20 \mu\text{g ml}^{-1}$), biotin ($1 \mu\text{g ml}^{-1}$) and nalidixic acid

($30 \mu\text{g ml}^{-1}$) for χ 1776. The majority of animals challenged i.p. with the DO-11 strain had viable bacteria in their organs 5 d later (Table 2). The i.v. group had fewer organisms on day 2 and none were recovered on day 5. In contrast, the χ 1776 strain, which was able to kill mice at a similar frequency as DO-11, could be isolated only within 2 d of injection, and was not cultured from any of the 19 surviving animals examined on day 5. These findings indicate that *E. coli* K12 DO-11 can survive and propagate in tissues of surviving animals. The χ 1776 strain seems to be less capable of prolonged survival, although it is just as virulent when judged on the basis of mortality.

It is apparent that *E. coli* K12 is relatively non-pathogenic. Very high doses of the organism are required in order to kill experimental mice. Its pathogenic potential is 10^3 - to 10^4 -fold less than wild type *E. coli*: using these same methods we find that the LD_{50} of a naturally occurring *E. coli* strain (isolated from the blood of a human patient) is 10^5 whereas that of K12 is 10^8 to 10^9 . Although χ 1776 cannot colonise the intestine of conventional or germ-free animals, it is equally virulent as *E. coli* K12 DO-11 when assayed by these methods in mice.

These results provide the basis for an assay for the possible increased virulence of *E. coli* strains into which foreign DNA has been inserted. The LD_{50} on parenteral challenge of the parental and recombinant strains could be compared, and virulence determined regardless of either strain's ability to colonise the intestinal tract. The criteria for pathogenicity would be acute mortality in terms of the numbers of deaths following the challenge dose and survival *in vivo* of the test organism in terms of the number of viable bacteria and length of time they are recovered in the organs of surviving animals. In this regard, χ 1776 was equally virulent as DO-11, but it did not survive as well as the conventional K12 strain, indicating that its genetic constitution rendered it debilitated *in vivo* and more susceptible to host defence mechanisms.

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Inability of debilitated *Escherichia coli* χ 1776 to colonise germ-free rodents

The potential hazards of recombinant DNA research have focused attention on the development of a strain of bacteria that will not survive outside the laboratory. A genetically engineered strain of *Escherichia coli* K-12 has been developed that not only has several growth requirements (including diaminopimelic acid), but also exhibits high sensitivity to ultraviolet radiation, bile salts, detergents, antibiotics and temperature. This debilitated strain has been designated *E. coli* χ 1776 and has been widely recommended for use in recombinant DNA research¹. So far only conventional animals have been used to test the ability of a genetically engineered bacterium to replicate *in vivo*. We postulated that lack of competition from the normal bacterial flora, due to disease state or to antibiotic therapy, might increase susceptibility to colonisation by *E. coli* χ 1776. The germ-free animal represents an extreme state in which resistance to bacterial colonisation is decreased by lack of indigenous microbial competition and by an underdeveloped immune system². We therefore attempted to colonise germ-free rats and mice (maintained at the University of Wisconsin, Gnotobiotic Laboratory) with *E. coli* χ 1776. Each of several attempts to colonise the gastrointestinal tract was unsuccessful; viable organisms could not be cultured from either fecal material or tissue samples for up to 2 months after inoculation.

To assess the ability of χ 1776 to survive in fresh fecal samples and to obtain an indication of the sensitivity of our assay techniques, the following experiment was performed. Germ-free colon contents (2g) were mixed with 2 ml of a broth culture of χ 1776; after 1 h of incubation at 37 °C, the number of viable organisms of χ 1776 decreased from 5.5×10^7 to 1.8×10^7 per ml; after 24 h at 37 °C, this mixture contained 6.0×10^5 organisms per ml. Thus fecal material was not excessively toxic to χ 1776, and our assay techniques could detect viable χ 1776. Twelve germ-free Sprague-Dawley rats were inoculated by way of oral cavity, the drinking water and the food and bedding. Aliquots of all cultures and swabs (used for oral cavity inoculation) yielded χ 1776 after they were removed from the germ-free isolator and cultured onto appropriate bacteriological media. Fresh fecal samples were collected on days 1, 2, 3, 4, 7 and 10 after inoculation and within 1 h were plated onto bacteriological media. On day 10, two animals were killed and various organs and tissues were homogenised. The homogenate was serially diluted in buffered saline with gelatin³, plated and incubated both aerobically and anaerobically. No growth was observed in the oral cavity (swab), or in tissue and contents from the stomach, duodenum, ileum, caecum, colon, lungs, trachea or bladder of the animals tested. In addition, these rats had maintained the enlarged caeca characteristic of germ-free rodents.

In the next experiment ten germ-free rats were inoculated with a 24-h broth culture of *E. coli* χ 1776 (5.5×10^7 viable organisms per ml) as follows. Two rats received 1 ml of culture rectally to bypass the acid conditions of the stomach; two rats were swabbed orally to repeat the conditions of the previous experiment; three rats received 1–1.5 ml of broth orally in an attempt to increase the oral dose; and three rats were given 450 ml of drinking water containing 50 ml of the 24-h broth culture and 400 ml of diaminopimelic-acid-supplemented (100 μ g per ml) water. Fresh fecal samples were collected on days 1, 3, 7, 11, 25, 31 and 60 after inoculation. All fecal cultures were sterile. Two rats were killed on day 11 (one rat had received 1–1.5 ml of broth culture orally and one had received the contaminated drinking water). Organs and tissues were analysed for bacterial contamination as in the previous experiment. No growth was observed in the oral cavity or in any of the tissues or gut contents of the experimental animals, which again had the enlarged caeca characteristic of germ-free rodents.

In another experiment, two germ-free BALB/c mice were given the 24-h broth culture of χ 1776 in their drinking water after each was inoculated by swabbing the oral cavity with a sample of the broth culture. Fresh fecal samples were collected on days 1, 2, 3, 5 and 7 after inoculation. All fecal cultures yielded no bacterial growth, although the drinking water (supplemented with a broth culture of χ 1776) remained culture-positive throughout the experiment (7 d).

In a further attempt to increase the initial challenge dose we anaesthetised rats and gave them viable organisms through a stomach tube. In the first experiment, six germ-free rats were inoculated either intragastrically or both intragastrically and intrarectally with 1 ml of a 48-h broth culture containing 7.2×10^7 viable units of χ 1776 and about 10^3 viable units of *Staphylococcus epidermidis* per ml (an accidental laboratory contaminant). Fresh fecal samples were cultured 24, 48 and 72 h after inoculation. No viable χ 1776 organisms were recovered, but high numbers (10^{10} per g dry weight) of *S. epidermidis* were recovered consistently. In the second attempt to introduce χ 1776 by stomach tube, six germ-free rats received 1.0×10^8 viable organisms in 5 ml of a 24-h broth culture. Fecal samples collected on days 1, 2, 5 and 11 after inoculation yielded no bacterial growth. One death, attributed to anaesthesia, occurred during the first 24 h after inoculation. Viable χ 1776 organisms were obtained from the stomach and duodenum of this animal, while the ileum, caecum and colon remained sterile. Other animals were killed (one on day 2 and two on day 11) and the contents of the various regions of the gastrointestinal tract yielded no viable organisms when cultured.

In our laboratory, *E. coli* K-12 (obtained from Dr O. Smithies, University of Wisconsin) easily colonised germ-free rats and mice. Germ-free animals were associated by inoculating the oral cavity with a swab that had been dipped in a broth culture containing 2.8×10^6 viable organisms per ml. *Escherichia coli* K-12 was established in pure culture: viable counts of 2.0×10^{10} organisms per g dry weight of caecal tissue and contents were observed within 24 h of inoculation.

It was surprising that *E. coli* χ 1776 failed to establish and replicate in germ-free rats and mice. After oral challenge, Blattner *et al.*⁴ cultured *E. coli* K-12 from feces of the conventional rat; a debilitated form of K-12 (DP50) certified for use in recombinant DNA research demonstrated some survival capacity in conventional rats, although this survival was significantly below the level of *E. coli* K-12 survival. The literature contains no report of an attempt to demonstrate *in vivo* survival of *E. coli* χ 1776 in an animal that lacks a competitive normal microbial flora. At the very least, we expected to recover low numbers of the organism from fecal samples during the first few days after inoculation. However, in all our attempts to colonise germ-free rats and mice, no viable organisms were recovered. A reason for the failure of χ 1776 to establish in germ-free animals may be the extreme bile sensitivity and/or the nutritional requirements of the organism. Survival of mutant bacteria in the gut might also be augmented by certain components of the microbial flora because debilitated microorganisms have been shown to persist for at least 24 h in the gut of conventional rats⁴.

This work with germ-free animals supports the contention that *E. coli* χ 1776 cannot survive outside the laboratory. The fact that it cannot colonise the gastrointestinal tract, even in the absence of a competitive normal flora supports the use of this microorganism in recombinant DNA research. Perhaps other bacterial strains recommended for such use should be assessed for their capacity to colonise or infect germ-free animals.

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Transformation of plasmid DNA into *Streptomyces* at high frequency

OVER 60% of known antibiotics¹ are produced by *Streptomyces* species, including many substances with valuable clinical and other applications; they therefore have considerable medical, biological and commercial importance. Although the process of gene exchange mediated by conjugation within these actinomycetes is apparently widespread², the transfer of genetic material between individuals by such sexual means is, by definition, predominantly restricted to members of the same species. Transfer of genes as free DNA (transformation) or by viral mediation (transduction) has been well documented in some microorganisms^{3–5}, but the efficiency of these processes also falls dramatically with increasing divergence of the species involved, as the homology of their DNA sequences decreases. In any case, the occurrence of these processes in streptomycetes remains to be established. This situation has hitherto precluded the generation of new combinations of DNA sequences from diverse actinomycete species which could have profoundly beneficial effects on both the range of chemical structure and the quantity of antibiotics produced⁶. We report here the development of a plasmid transformation system for *Streptomyces* which should allow the cloning of any DNA sequence into these organisms and which, potentially, provides a means of directly manipulating the pathways of antibiotic production. The system involves the uptake of covalently closed circular (ccc) DNA by protoplasts in the presence of polyethylene glycol (PEG) and the visual detection of transformants, at high resolution, after regeneration of the protoplasts.

The *Streptomyces coelicolor* A3(2) plasmid SCP2* (ref. 7) was used as transforming DNA and as a potential cloning vector. This self-transmissible plasmid, the first example of a physically and genetically characterised streptomycete sex factor, is a ccc DNA molecule of molecular weight 18×10^6 (ref. 8). When grown in contact with a confluent culture ('lawn') of an SCP2⁻ strain, isolates carrying SCP2* are surrounded by a narrow zone in which the development of the SCP2⁻ culture is inhibited⁷. This inhibition requires hyphal contact leading to conjugation between the two types of strain and may have features in common with lethal zygotis in *Escherichia coli* in which F⁻ cells are often killed when involved in multiple mating reactions with strains carrying the F factor in the integrated (Hfr) state^{8,9}. Reconstruction experiments indicated that this phenotype allowed the recognition of SCP2*-containing spores in an SCP2⁻ population at a frequency of $\leq 10^{-9}$ and thus should provide a powerful system for the detection of cells transformed with naked SCP2* DNA. SCP2* can be transferred by mating from *S. coelicolor* A3(2) to *S. parvulus* ATCC 12434 and the 'lethal zygotis' phenomenon is shown by the resulting SCP2* *S. parvulus* strains against SCP2⁻ *S. parvulus*¹⁰.

Cultures of *S. virginiae* become competent for DNA uptake in the early stationary phase and this competence has been exploited to transfect the mycelium with actinophage DNA¹¹. However, other species, including *S. coelicolor* A3(2), failed to show competence. The infection of protoplasts of *S. kanamy-*

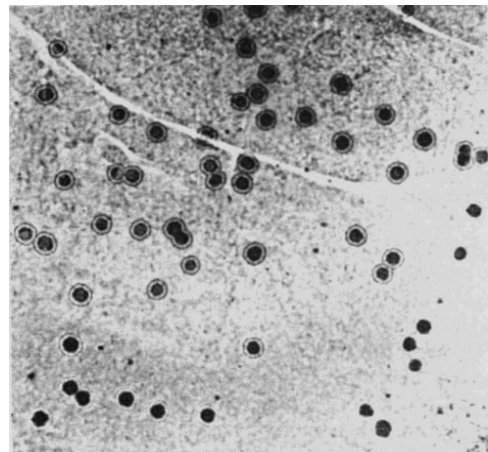


Fig. 1 SCP2* determined 'lethal zygotis' in *S. coelicolor*. Centres of lethal zygotis resulting from the outgrowth of single SCP2*-containing spores in a confluent lawn of SCP2⁻ strain M130 (*hisA1 uraA1 strA1*). Magnification $\times 2$.

ceticus with actinophage DNA¹² and the enhancing effect of PEG on the transformation of *E. coli* spheroplasts with DNA of the bacteriophage Φ X174 (ref. 13) have been reported. We therefore attempted to transform PEG-treated protoplasts of derivatives of *S. coelicolor* A3(2) and *S. parvulus* ATCC 12434 with SCP2* DNA, and to detect the transformants by regenerating the protoplasts and assaying spores from the resulting culture for their ability to show SCP2*-determined lethal zygotis within a confluent SCP2⁻ lawn.

Covalently closed circular SCP2* DNA was prepared by the large-scale method described by M. J. B., D. A. H. and R. F. Freeman⁷, from cultures of *S. coelicolor* A3(2) derivatives containing the sex factor; the DNA was demonstrated to be free of viable bacteria by spreading samples on plates of supplemented R2 medium. Protoplasts of strains of *S. coelicolor* A3(2) and *S. parvulus* ATCC 12434 were prepared as previously¹⁴, but without lytic enzyme no. 2 in the lytic solution. All subsequent steps, up to plating, were carried out at room temperature. For each transformation assay approximately 2×10^7 protoplasts (estimated by viable counts and containing $<0.1\%$ non-protoplasted units as determined by plating samples of the protoplast preparation after dilution in water¹⁴) were pelleted by centrifugation at 1,000g for 7 min; the supernatant was removed and the protoplasts were gently resuspended in the minute volume of buffer remaining with the pellet. To each aliquot of protoplasts, 20 μ l of sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing varying amounts of SCP2* DNA were added with gentle mixing. 0.5 ml of varying concentrations of PEG 1000 (Koch-Light) in P medium¹⁴ were added, followed 1 min later by an equal volume of a PEG 1000 solution at half the original concentration and, after a further 3 min, 4 ml of medium P. (Note that the dimethyl sulphoxide present in the PEG solution used for protoplast fusion¹⁴ was omitted.) The suspensions were centrifuged at 1,000g for 7 min; each pellet was resuspended in 0.2 ml of medium P and the samples were pipetted on to plates of R2 regeneration medium¹⁴ supplemented with auxotrophic growth requirements of the recipient strain and gently spread with a wire loop. Serial dilutions of some of the transformation mixtures were also made to obtain accurate estimates of the frequency of DNA uptake.

After incubation of the regeneration plates for 10 d at 30 °C, spores were collected from the confluent cultures¹⁵ and serial dilutions of the spore suspensions were spread on to appropriately supplemented plates of R2 medium which had also received enough spores of an SCP2⁻ isolate to produce a confluent lawn. In this way, SCP2*-containing spores could be readily detected by their ability to elicit lethal zygotis (Fig. 1). Where serial dilutions of the transformation mixture gave rise

to single colonies, these were picked to patch-plates and assayed for their ability to produce SCP2*-determined lethal zygosis by later replicating the patches to an SCP2⁻ lawn spread on an appropriately supplemented R2 medium plate. Several presumptive SCP2* transformants were purified and attempts made to isolate ccc DNA from them. All yielded ccc DNA which proved to be indistinguishable from SCP2* by restriction endonuclease analysis with a mixture of the enzymes *EcoRI* and *HindIII*, which cleaves the plasmid into two fragments of unequal size⁷. These results demonstrated unambiguously that transformation by SCP2* DNA had occurred.

The effect of PEG concentration on the frequency of transformation of *S. coelicolor* A3(2) by SCP2* DNA is shown in Fig. 2. A concentration of 20% (w/v) seemed to be optimal, although only slightly lower levels were obtained at higher concentrations; no reproducible transformation was obtained in the absence of PEG. All subsequent experiments were therefore carried out with 20% PEG.

The effect of DNA concentration on the frequency of transformation of *S. coelicolor* A3(2) is shown in Fig. 3. The yield of transformants was apparently proportional to a wide range of SCP2* DNA concentrations up to at least 2×10^{11} SCP2* molecules ml⁻¹ ($5.6 \mu\text{g ml}^{-1}$), which represents a plasmid

Fig. 2 Effect of PEG concentration on transformation of *S. coelicolor* A3(2) with SCP2* DNA. SCP2* DNA was added to 10^7 protoplasts of strain M130 to a final concentration of 2×10^{11} molecules ml⁻¹ at various PEG 1000 concentrations. The regenerated protoplasts were assayed for the presence of SCP2* as described in the text. The results are expressed as the number of SCP2*-containing spores recovered after regeneration and enrichment per 10^7 original viable protoplasts. ●, Mean results obtained from three experiments using PEG 1000 concentrations of 1.5–50%. ○, Mean results obtained from two experiments using PEG 1000 concentrations of 5–25%.

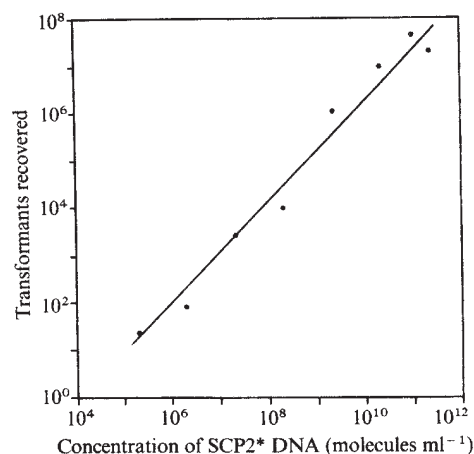
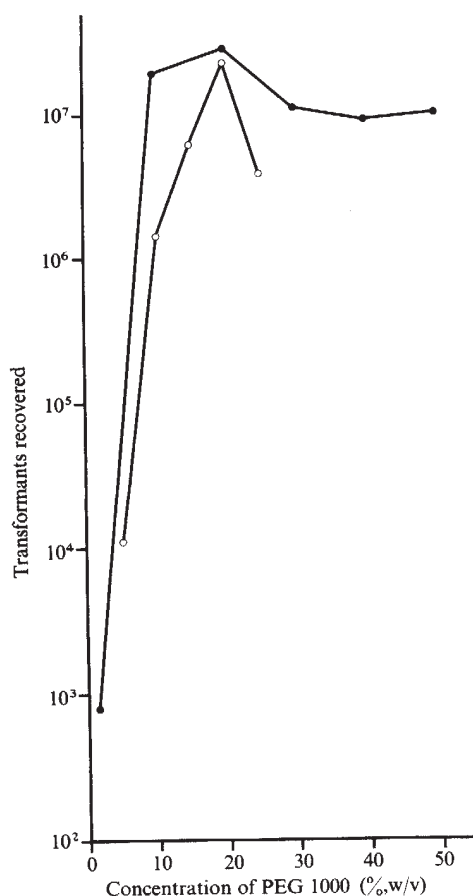


Fig. 3 Effect of DNA concentration on transformation. 2×10^7 protoplasts of *S. coelicolor* derivative M130 were treated with various concentrations of SCP2* DNA in the presence of 20% PEG. Regenerated protoplasts were assayed for the presence of SCP2* as described in the text. The results are expressed as the number of SCP2*-containing spores recovered after regeneration and enrichment per 2×10^7 original viable protoplasts.

molecule: protoplast ratio of 5,000. Transformation was not detected at DNA concentrations of less than 10^5 molecules ml⁻¹.

Reconstruction experiments indicated that an enrichment for the self-transmissible plasmid of up to 10^4 occurs when SCP2*-containing protoplasts are regenerated and grown in a predominantly SCP2⁻ protoplast lawn. Because of this enrichment factor, and the possibility of a variable loss of viability of protoplasts during the transformation procedure, the true proportion of transformed protoplasts was determined in experiments in which either individual colonies derived from single regenerated protoplasts or spores from confluent lawns of dilutions of the transformation mixtures which originated from a known number of treated viable protoplasts were assayed for their ability to elicit lethal zygosis. The results obtained using DNA concentrations of 2×10^{11} molecules ml⁻¹ indicated remarkably high transformation frequencies: 4–20% for *S. coelicolor* A3(2) and >0.1% for *S. parvulus* (a proportion of the spores collected from a lawn of less than 10^3 regenerated protoplasts of *S. parvulus* had acquired SCP2*, indicating a transformation frequency of at least 1 in 10^3 protoplasts).

The mechanism by which PEG induces transformation is not known. It may interact with the cell membrane to make it more permeable to DNA. Alternatively, because nucleic acid molecules have been shown to adopt compact forms in solutions containing high concentrations of PEG¹⁶, it is possible that the stimulation results from a conformational change in the DNA molecules facilitating penetration into the protoplasts.

These results describe a very efficient method for achieving and detecting the transformation of both *S. coelicolor* A3(2) and *S. parvulus* ATCC 12434 with plasmid DNA. A restriction endonuclease cleavage map of SCP2* (refs 7, 17) indicates the presence of single, well separated recognition sites for both the enzymes *EcoRI* and *HindIII*. It is likely that one or both of these sites will prove amenable to the insertion of DNA sequences from any source using the recent developments in recombinant DNA technology¹⁸ without damaging functions essential for plasmid replication. The presence of multiple sites for the restriction endonucleases *BamHI* and *SalPI* (an isoschizomer of *PstI*¹⁹) may facilitate the construction of smaller derivatives of SCP2* which would possibly be more suitable as future cloning vectors. Further, these techniques may possibly be used in the transformation of many other *Streptomyces*

species, and may therefore prove to be of considerable value in the development of cloning systems for these important industrial prokaryotes.

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A chymotrypsin-sensitive step in the development of *Dictyostelium discoideum*

THE cellular slime mould, *Dictyostelium discoideum*, is an ideal organism with which to study the changes in cell behaviour resulting from the formation of cell–cell contacts (ref. 1 and for review, ref. 2). The role of adhesive interactions in the development of this organism is understood in outline and much is known about the nature and developmental kinetics of the macromolecules thought to be involved in establishing stable cell–cell contacts during aggregation³. There is now good evidence that the formation and maintenance of contact between amoebae is necessary for the synthesis of several developmentally regulated enzymes⁴ and several polypeptides characteristic of post-aggregation cells⁵. Clearly, it is important to understand the molecular mechanisms involved in cell–cell interactions, as they not only make multicellular aggregates structurally coherent, but also induce the synthesis of developmental gene products. In our attempts to identify the cell surface structures responsible for adhesion and developmental triggering, we have allowed starving amoebae to develop in the presence of proteolytic enzymes that might modify accessible cell surface proteins. We report here that in the presence of α -chymotrypsin amoebae are able to undergo normal aggregation and cell–cell adhesion but are unable to differentiate into stalks or spores.

Experiments were carried out using *D. discoideum*, NC4, grown on *Escherichia coli*, B/r (ref. 6). Cells, washed free from bacteria, were plated on Millipore filters (5×10^7 cells per filter) at pH 7.5 in the presence of α -chymotrypsin (Sigma Type II; 3 mg per ml of solution). The amoebae displayed normal chemotactic behaviour and formed tight aggregates, but further development was blocked at this stage. The adhesiveness⁷ of these cells was normal (Table 1). Electron micrographs showed that α -chymotrypsin-blocked cells had not begun to differentiate as no characteristic prespore (or prestalk) structures could be seen, even after 24 h. Prespore vesicles are normally visible in posterior sections of slugs formed after 12 h of development.

We think that the effect is due to α -chymotrypsin activity rather than a contaminant of the enzyme. Neither trypsin nor

chymotrypsinogen A at 3 mg per ml had any effect on development, but when mixed they had a similar blocking effect to that of α -chymotrypsin. Active chymotrypsin is formed from its precursor chymotrypsinogen A by the proteolytic action of trypsin.

D. discoideum can rapidly recapitulate developmental events when pre-culmination cell masses are disaggregated and replaced on a solid support⁸. We have tested the ability of cells to evade the blocking effect of α -chymotrypsin by allowing them to develop normally for periods of up to 10 h before disaggregating them and replating in the presence of the enzyme. Cells that had been allowed to form aggregation streams before disturbance developed into morphologically normal fruiting bodies whereas cells disturbed at an earlier stage were still susceptible to the enzyme. These results suggest that cells stimulated to enter the developmental pathway, presumably as a result of contact formation, do not require continued expression of the α -chymotrypsin-sensitive components and retain their developmental commitment for several hours.

Table 1 Intercellular adhesiveness in *D. discoideum*

Treatment	Collision efficiency %
Control	14.72 $\pm$ 1.06
Control + EDTA (10 mM)	11.36 $\pm$ 0.80
α -Chymotrypsin (3 mg per ml)	16.62 $\pm$ 2.09
α -Chymotrypsin + EDTA	13.81 $\pm$ 1.13

Control material consisted of NC4 cells allowed to develop in the absence of bacteria for 8 h. Single cell suspensions were obtained by vortex agitation for 15 s in sodium phosphate buffer (20 mM, pH 7.5) or where appropriate, in buffer containing 10 mM EDTA. The cell concentration was adjusted to 1×10^6 particles cm^{-3} and suspensions aggregated at 20 °C in a Couette viscometer for 20 min. Samples were taken at 5-min intervals, counted by haemocytometry and the collision efficiency calculated for each cell population. Experiments were repeated at least three times. No adhesive differences were observed between cells grown in the presence of α -chymotrypsin and those merely exposed to the enzyme for 15 min before the adhesion assay. These results were therefore pooled to give the figure in the table. Adhesiveness is expressed as collision efficiency % $\pm$ s.e.m. A shear rate of 10 s^{-1} was used throughout. Application of the Student's *t* test shows that there is no significant difference in adhesiveness between the control and the α -chymotrypsin-treated cells ($P > 0.01$), the control + EDTA ($P > 0.01$), or the α -chymotrypsin + EDTA ($P > 0.01$) populations. However, EDTA slightly reduced adhesiveness.

Several explanations of the α -chymotrypsin effect occur to us. The enzyme may be destroying a protein present at the cell surface which is distinct from the contact sites A but involved in post-aggregative signalling⁹. Whether this putative component requires normal functioning of the contact sites is not known. Alternatively, the short-range cellular interaction required for entry into the prespore pathway¹⁰ might involve a diffusible component susceptible to α -chymotrypsin attack, although the low molecular weight factor (helper) required for entry into the stalk pathway¹¹ would have to be similarly affected, as neither prespore nor stalk cells are formed from α -chymotrypsin-blocked amoebae.

Town and Gross⁹ have suggested that interruption of post-aggregative signalling, involving cyclic AMP, may be responsible for the effects on polypeptide⁵ and enzyme synthesis⁴ that occur after mechanical disruption of cell aggregates. It is possible that the effect of α -chymotrypsin may be to impair the functioning of this cyclic AMP-dependent post-aggregative signalling.

Whatever the explanation of the effect we describe, our results suggest that it is not the formation of stable cell–cell contacts as such that stimulates differentiation in amoebae of *D. discoideum* but simultaneous or subsequent involvement of components sensitive to digestion by exogenously applied α -chymotrypsin.

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Charge separation as a component of the structural requirements for hormone activity

THE establishment of a theory which would satisfactorily predict the relationship between molecular structure and plant hormone activity would be of major importance in improving our fundamental knowledge of plant growth regulation and would be of great practical significance to the agrochemical industry. The subject of auxin structure–activity relationships has been widely reviewed^{1–7} but each theory has been found, in some respect, defective. Techniques of theoretical chemistry, biochemistry and plant physiology are used here to draw attention to the probability that charge separation as a component of the structural requirements for auxin activity has been prematurely⁶ rejected.

During studies of the auxin requirements of English sycamore (*Acer pseudoplatanus*, L.) cell suspension cultures, we noted that the relative activities of a range of auxins in supporting culture growth (by promotion of cell division and cell enlargement) were very similar to the relative activities of the same compounds in the promotion of cell enlargement in the pea curvature test⁸ (Table 1). This confirmed previous observations^{5,9} that substances which have auxin activity in one assay system usually have activity in all other systems (although relative activities differ, probably due to differences in the rate of penetration into, and metabolism by, different tissues). Recognition of this fact led Thimann to propose⁹ his ‘master reaction’ theory, which implies that auxins have a unifying structural feature which permits them to interact with a receptor molecule, thus triggering the various metabolic responses.

Thimann and Porter¹⁰ concluded that the primary growth action exerted by auxins depends on the presence of a fractional positive charge on a planar lipophilic nucleus at about 5.5 Å (0.55 nm) from a negative carboxyl oxygen. In the case of indol-3-yl-acetic acid the fractional positive charge was believed to lie on the indole ring nitrogen. This charge separation theory¹⁰ was discounted by Kaethner⁶ in his presentation of the conformational change theory. Also, although it had previously received widespread approval it was in any case challenged by the discovery that indol-1-yl-acetic acid had high activity (refs 3, 12 and P. E. Pilet, personal communication) and that indol-4-yl-acetic acid had low but real activity¹². The former compound was calculated to have a distance of 3.2 Å (0.32 nm) between the carboxylate oxygen and the indole ring nitrogen atom, and the latter was found to have a distance of

7.3 Å (0.73 nm) between the corresponding charges. Several other clear exceptions to the theory have been noted by Thimann¹² and others. The use of self-consistent field molecular orbital calculations¹³ has permitted us to check π and σ contributions to net atomic charges and also to determine probable optimum geometrical configurations of active molecules by considering the total energy of various conformations. Clearly, conformation is a critical parameter in establishing any charge–distance relationship in a molecule where bond rotation can occur. To establish the most probable conformation of the side chain of the natural auxin indol-3-yl-acetic acid, the energy of the whole molecule was calculated as the side chain was rotated through 180°, about the C₃ ring carbon–methylene carbon axis. The conformation in which the ring plane was perpendicular to the C₃ ring carbon–methylene carbon–carboxylate carbon plane proved to be the most energetically favourable by about 10×10^3 J mol⁻¹. This conformation is consistent with Veldstra's conclusion² that the ability of the side chain to project out of the plane of the ring is a requirement for auxin activity, the perpendicular conformation being the most favourable.

The Thimann and Porter theory¹⁰ asserted that the indole ring nitrogen of indol-3-yl-acetic acid bears a fractional net positive charge. Figure 1 shows the total net atomic electron density for the ring atoms of indol-3-yl-acetic acid as determined by self-consistent field molecular orbital calculations. The calculations predict that the nitrogen atom bears a substantial net negative charge of -0.14 atomic units. This is due to the inductive effect on the σ bonds of the nitrogen atom, as it is more electronegative than carbon. Although there is a withdrawal of π electrons from the nitrogen atom, due to delocalisation into the conjugated ring system, this is outweighed by the inductive effect, resulting in the total net negative charge. These results concur with those of Bloor and Breen for indole¹⁴.

Self-consistent field molecular orbital calculations of total net atomic electron density for the ring atoms of a wide range of auxins were carried out; some examples are given in Fig. 1. This clearly shows that one unifying feature of the heterogeneous group of compounds is the presence of a fractional net positive charge at an atom on the ring separated by about 5 Å (0.5 nm) from the carboxyl oxygens when the side chain is in the thermodynamically favoured perpendicular conformation. The figure draws attention not only to charge separation as one particular unifying feature of the molecules but also makes clear the remarkable stereochemical similarities between the two members of the highly active indol-3-yl-acetic acid/indol-1-yl-acetic acid pair and between the two members of the weakly active indol-4-yl-acetic acid/phenylacetic acid pair.

Table 1 Comparison of the ability of a series of auxins to support sycamore cell suspension culture growth with their capacity to promote cell enlargement in the pea curvature test

Auxin	Test	
	Sycamore cell suspension culture growth*	Split pea stem curvature†
2,4-Dichlorophenoxyacetic acid	1.99	1,200
Naphth-2-yloxy-acetic acid	1.03	100
Naphth-1-yl-acetic acid	0.73	370
Indol-3-yl-acetic acid	0.42	100
Naphth-2-yl-acetic acid	0.34	
Phenylacetic acid	0.05	4

* Cell numbers (in millions per ml minus no-auxin-controls) after 21 d in the second passage in culture medium¹⁷ containing the specified auxin. Mean value for concentrations in the range 4.53–45.3 μ M.

† Activity in the split pea curvature test relative to indol-3-yl-acetic acid (100%). The concentration required to reduce outward curvature by 100° is divided by the concentration of indol-3-yl-acetic acid required to produce the same effect, and the fraction is multiplied by 100. Data from Porter⁸.

Our results strongly suggest that it would be premature to ignore charge separation as a component of the structural requirements for auxin activity. We propose a modification of Thimann and Porter's theory, namely, that a fractional net positive charge on a lipophilic nucleus at above 5 Å (0.5 nm) from an acidic group contributes to the reversible binding of an auxin to its receptor. The location of the positively charged site at a bridge carbon between two rings in the bicyclic auxins precludes the idea of a nucleophilic substitution reaction at this position.

Kaethner⁶ proposed that the ability of the side chain of a molecule to switch from a planar to a perpendicular conformation relative to the ring system is critical for auxin activity. This conformational change theory could explain the inactivity of certain di-*ortho* substituted auxins, such as the di-*ortho* substituted phenoxyacetic acids¹⁵, and the anti-auxin activity of certain isobutyric acids¹⁶. However, the fact that di-*ortho* substitution in the phenylacetic acids results in a substantial increase in activity, as shown by the high activity of 2,3,6-trichlorophenylacetic acid^{5,8}, seems surprising if the 'recognition conformation' is planar. Self-consistent field

molecular orbital calculations carried out for phenylacetic acid indicate that, as in the case of indol-3-yl-acetic acid, the preferred conformation is that in which the C₁ ring carbon-methylene carbon-carboxyl carbon plane is perpendicular to the ring plane (the 'modulation conformation' of Kaethner). The calculated energy difference between this conformation and that in which the C₁ ring carbon, methylene carbon and carboxyl carbon atoms are all in the ring plane, with the carboxyl oxygens staggered with respect to this plane (Kaethner's 'recognition conformation'), is about $13 \times 10^3 \text{ J mol}^{-1}$. Assuming a Boltzmann distribution, only about 0.5% of the molecules in a population would have the conformation required for them to be 'recognised'. Di-*ortho* chlorine substitutions relative to the side chain would be expected to decrease further the stability of the planar conformation due to unfavourable interactions between the carboxyl oxygens and the *ortho*-chlorine atoms. Clearly, in a population of these molecules a vanishingly small proportion would be in the 'recognition conformation'. Further quantitative information of the type given here is therefore required before a critical evaluation of any structure-activity theory can be made. For the moment, a consideration of the stereochemistry of active and inactive molecules suggests that there is no need to invoke a 'recognition conformation', because the differing effects between auxin series of substitutions di-*ortho* to the side chain are explicable in terms of the ability of the molecule to adopt an energetically favourable conformation in which the side chain projects out of the ring plane.

The physico-chemical characteristics of the auxin molecules suggest that reversible binding, involving van der Waals, hydrophobic and electrostatic interactions are likely to be involved in the binding of an auxin to its receptor molecule. Although the presence of a positive site at 5 Å (0.5 nm) from the carboxyl oxygens would contribute to the proposed binding interaction, other factors such as the lipophilicity of the nucleus and stereochemical effects are likely to be involved in determining auxin activity, inactivity and auxin antagonism.

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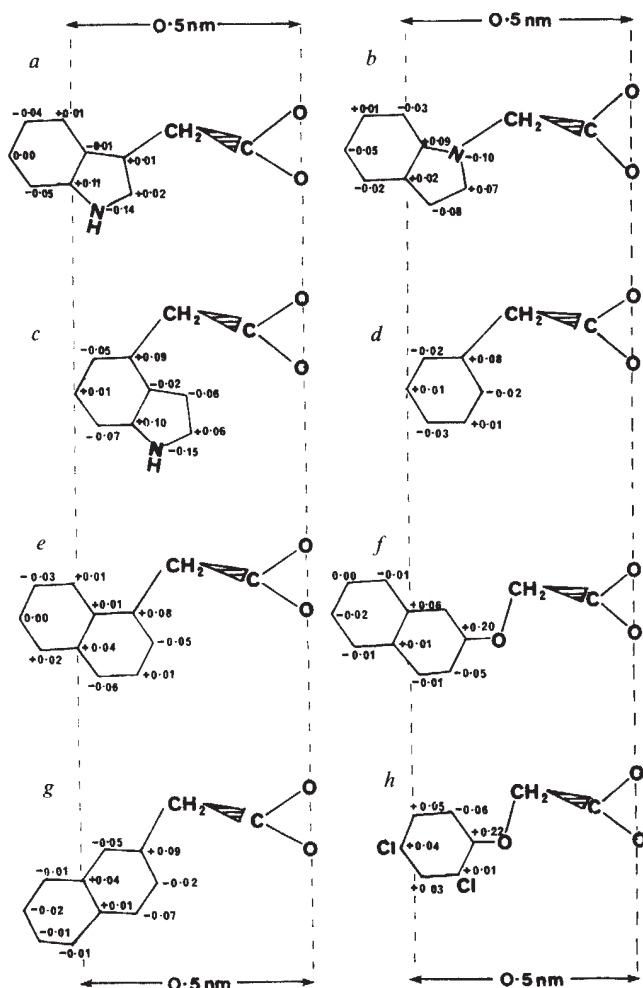
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Fig. 1 Total net atomic electron densities on the ring atoms of a range of auxins as predicted by self-consistent field molecular orbital calculations. The 0.5-nm distance between the oxygens of the carboxyl group and a positive site on the ring (when the side chain is in the thermodynamically favoured perpendicular conformation) is also shown. *a*, Indol-3-yl-acetic acid; *b*, indol-1-yl-acetic acid; *c*, indol-4-yl-acetic acid; *d*, phenylacetic acid; *e*, naphth-1-yl-acetic acid; *f*, naphth-2-yl-oxyacetic acid; *g*, naphth-2-yl-acetic acid; *h*, 2,4-dichlorophenoxyacetic acid.



reviews

What then is time?

G. J. Whitrow

Time and Man. By L. R. B. Elton and H. Messel. Pp. 114. (Pergamon: Oxford, New York, Toronto and Paris, 1978.) Hardback £4.50; paperback £1.95.

THIS is a wide-ranging book written at a semi-popular level on a subject that has long puzzled and intrigued mankind. On the title page the authors include a famous quotation from St Augustine: "What then is time? If no-one asks me, I know: if I am asked to explain it, I do not know". In other words, we all of us have an intuition of time, but we find great difficulty in accounting for it and deciding what time actually is.

The authors begin with our experience of the passage of time. When we are infants this is first associated with rhythmic changes in our bodily condition and then with movement and speed. Later we gradually become aware of the succession of events and of the role of time in social behaviour through ideas such as punctuality. Later still we learn to associate time with the great questions of philosophy and religion, and we come to feel that time is something 'absolute' over which we have no control.

Mention might have been made in this first chapter of learning to tell the time by the clock, particularly as in the next chapter the authors introduce the operational definition of time in terms of numbers and a measuring device. This approach to the question of time is by no means universal, but is characteristic of certain civilisations, notably modern industrial civilisations in which peoples' lives are ruled by the clock because of the need to make precise timings.

The authors devote a chapter to various practical devices that are used for measuring time, culminating in the development of atomic clocks. They then go on to discuss the synchronisation of clocks at different places, pointing out that no signal is instantaneous and that this particularly affects the relationships between clocks in relative motion. Discussion of the velocity of light and its measurement is followed by an elementary account of the special theory of relativity and a neat derivation of the formula for time dilation. The experiment by Hafele, in which atomic clocks were flown round

the Earth both eastwards and westwards to test this formula, is described, but no mention is made of the gravitational effect that was also involved in this experiment.

In a chapter on the unidirectional nature of time, the authors describe the objections to the statistical explanation of time's arrow. After discussing time and the interactions of elementary particles, they go on to consider the Universe as a whole. They show that the most satisfactory resolution of Olbers's paradox of the dark night sky is through the concept of cosmical expansion. Hubble's law is described, and its simplest interpretation is shown to lead to a rough value of 18,000 Myr for the age of the universe.

A chapter that contains much of interest on geological time, biological evolution and biological rhythms precedes the final chapter in which the authors revert to the subject of "time

and man" with which the book began. Piaget's investigations on the development of the child's idea of time are described, followed by consideration of time and the adult mind, and a number of literary quotations relating to man's concern with time. In this chapter the authors make the point that philosophers who wish to go beyond physics in their investigation of time should at least take notice of where physics has got to.

The book ends with a useful list of books and references. Although in places it is inevitably somewhat superficial, this book contains much of interest and can be recommended as a useful and stimulating introduction to the general subject of time. □

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Applied group theory

Induced Representations in Crystals and Molecules (Point, Space and Non-rigid Molecule Groups). By S. L. Altmann. Pp. 369. (Academic: London, New York and San Francisco, 1978.) £18; \$32.25.

THE appearance of yet another book on applied group theory calls, as its author admits, for some justification. Dr Altmann claims that his book meets the need for a comprehensive and comprehensible account of certain mathematical techniques which "have recently been found important in the discussion of point groups (Altmann, 1963a) and non-rigid molecules (Altmann, 1967)". The modestly anonymous flyleaf claims that "this monograph provides a unified and rigorous treatment of the theory and its applications" and that "no single book has dealt with the subject so completely and in so much detail . . . The fully worked examples provided for each theoretical step will come as a welcome relief for those frustrated by the unintentional obscurities in many high level texts", and so on.

Unfortunately these appetising promises are not fulfilled by the quality of the text. In his very first paragraph the author describes the first four or

five chapters as only "fairly" self-contained, and his working definition of symmetry operations, so far from being "rigorous", is inexcusably sloppy. This does not matter much for the purely mathematical developments, but his provisional identification of symmetry operations as rigid displacements (translations, rotations, and so on) makes the transition from crystals to non-rigid molecules painful in the extreme. As for the "unintentional obscurities" to be found in the works of other authors, the wild proliferation of symbols in the later chapters of this book makes the arguments almost impossible to follow, and the "carefully worked examples", particularly that of the octahedral group, show just how much mess can be created by using a theoretical steam-hammer to crack an elementary problem.

The last chapter, on the symmetry groups of non-rigid molecules, is distressingly confused and confusing. It reminds one all too vividly of Dr Altmann's 1967 paper, which in its time clouded the subject with a fog which took theoretical spectroscopists some years to dispel. The main source of confusion is Dr Altmann's failure to appreciate the main purpose served by the concept of molecular symmetry in,

for example, high resolution spectroscopy. The central problem in this field is that of relating the symmetry of an overall molecular state to the symmetries of its electronic, vibrational, rotational, torsional and spin-wave functions. This end can only be achieved if one classifies all these wave functions according to the same symmetry group—namely, a group of operations which commute with the full molecular hamiltonian, not just the “Born-Oppenheimer” hamiltonian, which by itself merely determines the electronic wave function for a fixed nuclear configuration. The elements of Dr Altmann’s groups (in so far as one can determine their physical interpretation from the primitive diagrams and the associated discussion) apparently involve, in general, restorative rotations of the molecule as a whole a fact which renders them virtually useless for classifying the rotational levels of non-rigid molecules. As for Dr Altmann’s remark, in the preface, that the symmetry groups of molecules such as difluoroboron methyl “had to be done in the past by computer”, the group character table of the much more complex molecule boron trimethyl was first derived by hand in a few days, and Dr Altmann does not mention that he

doubted its correctness for nearly ten years after its publication in 1963.

The book is, unfortunately, riddled with misprints (a particularly glaring one is in equation 6.22 of chapter 20, which conflicts with 9.1 of the same chapter), spelling mistakes (“lose” is repeatedly misspelled as “loose”) and phrases such as (p 336) “thus satisfying all vibronic interactions” which are hardly calculated to inspire confidence in Dr Altmann’s understanding of molecular quantum mechanics. How (p 326) can two molecular configurations “correspond to the same values” of the angular momenta, one would like to know?

If, as the flyleaf anticipates, the book finds its way on to the shelves of research workers in theoretical and solid-state physics, theoretical chemistry and theoretical molecular spectroscopy, one can only hope that they will distinguish clearly between the relatively innocuous mathematics and the appallingly confused physical interpretation which Dr Altmann assigns to it.

H. C. Longuet-Higgins

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Current cancer research

The Story of Cancer: On its Nature, Causes and Control. By A. C. Braun. Pp. 308. (Addison-Wesley: Reading, Massachusetts and London, 1977.) Hardback \$19.50; paperback \$9.50.

PROFESSOR BRAUN wants to make a coherent story out of the chaotic literature of cancer research. In his own words, he wants to review “the major pieces of information that we have available in an attempt to solve the jigsaw puzzle that we know as cancer. If all of the required pieces have now been identified we need only to fit them into their proper places to gain an understanding of the nature of the puzzle, but if major parts are still missing then we will, of course, be unsuccessful.” The present book is an expanded and more detailed documentation of concepts introduced by Professor Braun in his previous two books, *The Biology of Cancer* (1974) and *The Cancer Problem* (1969), and there can be few people concerned with cancer research who would not find much of interest in the present book. It is short (276 pages, 24 typewritten lines per page) and, because it derives from four lectures delivered to a select group of

gifted high-school students, it would be fairly easy for a *Scientific American* reader to digest (although any high-school students who can understand it must be extremely select).

The central chapters review much experimental work, especially on plant tumours, which Professor Braun is intimately familiar with, and attempt to produce a unified account of neoplastic growth in plants, amphibians, mammals, and man; from these central chapters I learned a great deal of experimental detail, especially about plant tumours, that was new and interesting. The first and last chapters, however, give the impression of being tacked on later to cover detection, prevention, treatment and molecular biology; they contain some errors, obscurities or restricted perspectives. (Particularly, although some cancers are certainly caused by “chemical substances that have been identified as cancer-producing and which must be identified and removed from the environment”. Professor Braun should not say that 70% or more are thus caused; many, and perhaps most, cancers are caused by certain sexual habits, smoking habits, and gross aspects of diet rather than by contaminants.)

Returning to the central chapters, Professor Braun’s main thesis is that tumour cells do not differ importantly

from ordinary cells in their nucleotide sequence; all the genetic information can perfectly well remain unchanged, for the key events are, he believes, epigenetic. Tumour cells, he argues, differ from normal cells in the sort of way in which differentiated cell types differ from each other, and he illustrates this argument with examples of plant tumours, frog tumours, mammalian tumours, and certain human tumours.

He describes how teratomas so malignant that a single transplanted cell can cause a tumour may, if implanted into a growing embryo, differentiate into normal organs; how malignant human neuroblastoma cells can be induced to differentiate terminally into RBCs, and how particular viral functions can produce a malignant phenotype without altering the genetic information of the infected cell, and so on. He describes in great detail the epigenetic changes that enable plant tumours to proliferate, and the ways in which this neoplastic behaviour can be reversed.

However, there is an alternative way of trying to get order out of the mass of current cancer research; instead of widening the field to be viewed and unified to include all human, mammalian, amphibian and plant tumours, it might be more profitable to narrow it to include only the human carcinomas. Human carcinomas, which comprise 85% of human cancers, have many characteristics in common with each other, and may differ so fundamentally from other human tumours or from non-human tumours that analogies are misleading. For this reason, I regretted that the discussion of examples included little on human carcinomas, and did not, for example, even mention that gross aneuploidy commonly precedes malignancy.

Finally, there is an over-disparaging review of the potentialities of *in vitro* mutagenicity assays such as the Ames test, and much sarcastic rhetoric against those who believe that the heritable neoplastic changes in cells are irreversible. The rhetoric is not always convincing, especially as the “somatic mutation” hypothesis which is being attacked is not clearly defined. Thus, this book is not quite the “story of cancer” that I would have liked, but it is still stimulating and interesting. But, as current cancer research contains far too much meaningless detail, the more people who try to stand back and make a story of it the better.

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Transplantation immunology and pathology

Handbuch der Allgemeinen Pathologie. Volume 6, Part 8: Transplantation. Edited by J. W. Masshoff. Pp. 1070. (Springer: Berlin and New York, 1978.) DM425; \$187.

THE philosophy behind this great tome, which attempts to cover the genetic, immunological and pathological aspects of tissue and organ transplantation, is stated somewhat defensively in the preface. Thus, "The editors have often questioned the utility of this kind of review volume in a large handbook. The rapid progress of science will outstrip, as a rule, their work in editing and preparing contributions. Whoever wants to keep abreast of recent developments will find the latest publications in scientific periodicals. The fundamental facts of contemporary knowledge, however, can only be gathered from comprehensive presentations where the widespread results are collected, compared and evaluated. Here we see the role of our volume and the reason for its publication".

To what extent have the editors and their contributors succeeded in this endeavour? Thirty authors (some better known than others, and about half from Germany or Switzerland but writing in impeccable English) have prepared 17 review chapters of variable length dealing with the genetics and chemistry of histocompatibility antigens; phylogenetic and ontogenetic aspects; the cells taking part in graft rejection and the mechanisms of rejection; tolerance (both antigen and radiation induced); graft-versus-host reactions; the transplantation of cells such as blood and bone marrow; the grafting of skin, cornea, connective tissue and bone; immunosuppression by antibodies; and medications and their toxicity. Some of the chapters dwell on clinical problems, and pride of place (in terms of space) is given to the general pathology of the transplantation reaction in experimental and clinical organ grafts (C. R. Jerusalem and P. H. K. Jap), a chapter that occupies roughly one-fifth of the text. Second in the pecking order is the subject of histocompatibility in the form of three chapters on the major histocompatibility system in man (M. Jeannet), the lymphocyte-defined (sic) components of the major histocompatibility complex (Miriam Segall), and the biology and chemistry of major histocompatibility complex gene products (D. A. L. Davies and M. Hess).

The claim by the editors that there

is a place for a book of this type is no doubt justified, for reviews are widely scattered, congress proceedings can be ephemeral and lacking in depth, and most books set themselves much more limited objectives. In judging its usefulness one would want to be satisfied that the chapters are written with authority; that the book is reasonably up to date; and that coverage of topics is sufficiently complete. I am not sure that approval can be wholly unqualified on any of these counts.

Let us take the question of coverage first. Several topics are conspicuous by their absence or treated in desultory fashion. For example, one would like to have seen chapters on *in vitro* correlates of graft rejection and the immunological monitoring of patients, on maternal-foetal relationships, enhancement, and the use of xenografts. One wonders whether these omissions came about by accident or design. Nor does the book emerge with full marks so far as topicality is concerned. For reasons which were at least partly beyond the control of the editors (the original editor, W. Masshoff, having died when the first manuscripts began to come in) most of the contributions are far from up to date. Thus, references to 1976 publications are rare, and even 1975 references are regrettably sparse in most chapters. As a result the reader frequently cannot escape a feeling of *déjà vu*. To take but two examples: a table giving HLA antigens and their phenotypic frequencies relies on material published in 1974, and the tentative discussion of the effects of

previous blood transfusion on the clinical outcome of kidney transplantation depends on the very earliest indications in this field.

Other criticisms of the book include the considerable and only partly unavoidable overlap between some chapters, the use by some authors of wrong (p404) or misleading (p441) terminology, the reluctance of some contributors to quote other reviews and to chance their arm in "comparing and evaluating", and the disproportionate amount of space (roughly one-fifth of the total book) taken up by the indices, mainly by an author index the value of which must be questionable. Finally, and certainly for UK readers not least, there is the absolutely prohibitive price.

It should, nevertheless, be said that research workers interested in various facets of transplantation are likely to find some parts of this book very useful. Several chapters (especially those dealing with bone marrow transplantation, pathology and graft-versus-host reactions) are magnificently illustrated, minor errors are relatively rare and the quality of paper and print is excellent. Having taken the decision to proceed with publication, the new editors (H. Cottier and E. Grundmann) have clearly worked hard and selflessly to complete an enterprise initiated by their former colleague, to whose memory the volume is dedicated.

Leslie Brent

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Fourier transform infrared spectroscopy

Fourier Transform Infrared Spectroscopy: Applications to Chemical Systems. Vol. 1. Edited by J. R. Ferraro and L. J. Basile. Pp. 311. (Academic: New York, San Francisco and London, 1978.) \$25; £16.25.

INTENDED to be the first of a series on applications of Fourier transform infrared spectroscopy, this book covers topics ranging widely from polymers, to chromatography and finally to problems with nuclear reactors. Obviously, the depth of treatment is liable to be very variable, and this proves to be the case. Some of the chapters—for example, Green and Reedy on matrix isolation methods, Bates on emission spectroscopy, Griffiths on uses in conjunction with gas chromatography, and Lauer on high pressure interferometry—are quite well written and contain a fair amount of detail on both apparatus and

results. The other chapters do not fit into the scheme as well: in particular, chapter 2 on applications to polymers and macromolecules, did little to show the advantage of the technique.

The book suffers from the usual problems of compilations: for example, duplication of topics. Similar remarks on the spectra of liquids occupy considerable space in chapters 5 and 6. There are also one or two errors of fact, sometimes caused by a desire to oversell the product. Perhaps the best example of this is the statement on p216 that "In the early 1950s a group of investigators showed that the advantages claimed for interferometry by Fellgett (1958) could be realised".

In summary, this is a useful book but I doubt whether the subject matter will easily stretch to more volumes in a series.

Geoffrey Duxbury

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Bioinorganic chemistry

Bioinorganic Chemistry: An Introduction. By Ei-Ichiro Ochiai. Pp. 513. (Allyn and Bacon: London, 1978.) £16.95.

THE intention of this book is to provide an introduction to bioinorganic chemistry, a "discipline . . . rapidly bridging the gap between inorganic chemistry and biochemistry", according to its author. The book, however, fails to bridge this gap, and the tone is set by the introductory sections, comprising a self-contained outline of coordination chemistry and a brief summary of "topics from biochemistry". This neat separation of the inorganic from the biochemical is perhaps inevitable when setting out the principles of the two subjects. But where are the principles of the discipline of bioinorganic chemistry? Succeeding chapters do not disclose them.

Optical and magnetic properties of metalloporphyrins, oxygen-carrying pigments, and details of some synthetic model oxygen carriers, are presented; but the inorganic and physical chemistry is not used to shed any light on phenomena of interest to the biochemist, such as cooperativity or the widely different capacities of haemoproteins to bind oxygen or carbon monoxide. The role of cytochrome *c* oxidase in reducing oxygen is next introduced. Why do the haem centres of, say, this enzyme and myoglobin have such different chemical properties? What could be different about the coordination chemistry of these two sites that is important in determining biochemical function?

Opinion will differ about the relative importance of different problems but it must be part of the role of inorganic coordination chemistry to suggest possible answers to this type of question. One seeks in vain throughout this book for insights on such aspects. The author has, however, sensed the difficulty and provided a chapter on oxygenases, in which an attempt is made to group enzymes according to their common function. But this chapter is still merely a list of compounds and their properties, reminiscent indeed of some treatments of inorganic chemistry. The lists reappear when chemicals are classified under the name of the metal atom. The chapter on copper proteins and enzymes divides crisply into three sections headed "Biochemistry," "Inorganic Chemistry," and "Mechanisms".

The author's difficulty is a real one. Little is known with certainty about the

structures of the metal centres in many proteins. And how is one to guess at mechanisms? This is especially true of copper proteins, although the recent successful X-ray structure of the copper centre in plastocyanin immediately outdates the book. Can the tools of inorganic chemistry help to resolve problems of structure if the principles of the subject so poorly illuminate the mechanisms of action? The author clearly thinks so and there is much reference throughout to the potential for using inorganic ions as probes. It is here that the book is most successful, for example, in recounting the structural information obtained by the substitution of cobalt for the metal in zinc-containing proteins. It is pre-

sumably for this reason that much space is given over to discussion of optical and magnetic properties of metal centres.

The book is full in its coverage, gathering together much of the literature up to about 1974 or 1975. But it is surely not enough to gather the facts and to arrange them in a systematic way. Any successful book must provide insights and correlations drawn from this information. If the reader is left to do it for himself the book will have failed to fulfil its aim.

A. J. Thomson

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Colour and craftsmanship in psychopharmacology

Psychopharmacology: A Generation of Progress. Edited by M. A. Lipton, A. DiMascio and K. F. Killman. Pp. 1731. (Raven: New York, 1978.)

SUNBURST, Steeplechase, Goose in the Pond . . . this book inevitably reminds one of the great American tradition of the patchwork quilt. And a book like this, which gives itself the bold and resounding title of *A Generation of Progress*, clearly aims to produce a design and a total effect. The work is made up from 149 patches which have been contributed by 249 authors or joint authors, and it was stitched together by three editors and a nine-member *ad hoc* committee on behalf of the American College of Neuropsychopharmacology. Quite a sewing party.

In the event, and with all this multiple handiwork, does any sort of grand design in fact stand out? The answer is an unequivocal "yes". The book succeeds not only in terms of its having attracted many worthwhile individual contributions but also by an astonishing feat of editorship, which truly builds a picture of the large movements of twenty years and which identifies the very basic questions.

Ideas, models, and techniques and their mutual relationships are the recurrent themes. The book deals with matters at many levels from neuroanatomical and histochemical, through biochemical pharmacology, behavioural pharmacology, electrophysiological studies, clinical pharmacology and toxicology, the use of drugs in mental disorders, and problems of drug abuse. The relationships between these different levels of endeavour is made

real.

Some sections of the book are necessarily concerned with the jobbing business of factual review, whereas in other parts the authors have been given the task of looking more particularly at concepts. In several chapters there is a throw-away phrase (or grumble) about the "constraints of space", and authors have been kept within the bounds of very tight word limits. Sometimes this demand seems to have been defeating, and an author has not succeeded within his allotted few pages in producing a balanced summary of a research area which really did not lend itself to such compressed treatment. Very short contributions seem in fact generally to have been more successful when developing an idea rather than reviewing an objective area. Papers such as that by Seymour Kety on "Strategies of Basic Research", or a clutch of papers on the methodology of clinical research (with an essay by Joseph Zubin on the biometric approach to psychopharmacology) are particularly rewarding.

To be of value, any scientific review must be critical, and it is immediately evident which contributors to this book have actually read and evaluated the material they are presenting, and which have just taken everything at face value. Confidence is somewhat impaired when the names of referenced authors are woefully misspelt and papers quoted wrongly or irrelevantly.

But that is a criticism only of the odd patch or two. This is a book of enormous worth and importance which gives the live impression of colour and craftsmanship; it is not one of those machine made and commercial productions.

Griffith Edwards

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Poor introduction to insect control

Biochemical Insect Control: Its Impact on Economy, Environment and Natural Selection. By M. Sayeed Quraishi. Pp. 280. (Wiley Interscience: New York, Sydney and London, 1977.) £14.40; \$26.10.

THE subject matter of this book is not immediately obvious from the title. In fact the book is mostly devoted to control of insect pests by insecticides, although short sections dealing with alternative approaches, such as the use of chemosterilants and compounds with hormonal activity, are included. All these methods have biochemical aspects but these are not generally emphasised in this text. Furthermore, there is only brief mention of economic and environmental considerations, and reference to the impact of 'biochemical' insect control on natural selection in the title seems to be justified only by a very short section dealing with resistance to insecticides. This confusion over the title is symptomatic of a general fault throughout the book.

From the wide coverage attempted in this short book, it seems to be intended as an introduction to insect control. However, the account is often confusing and generally poorly organised, tending to preclude such use. In addition, there are many errors that are not only far too numerous for a scientific work, but would also set a very bad example for students entering this field.

Arrangement of material in the book does not seem to have been carefully thought out. This has led, in many cases, to unnecessary repetition or to the omission of essential information and explanations. The first section is titled "Chemicals for Insect Control". Except for an inappropriately placed chapter purportedly dealing with pharmacodynamics of chlorinated hydrocarbons and their present status, this section comprises subsections made up of chapters each concerned with a group of chemicals according to type or origin. Many of these chapters contain detailed discussion of mode of action, activation and degradation. However, later in the book there are chapters—for example, those in a subsection titled "Insect-Insecticide Interactions"—that deal specifically with these subjects. Another example of poor planning is the chapter on "Attractants, Repellants and Antifeedants". This is placed in the subsection "Synthetic Insect Control Agents" and yet contains discussion of chemicals such as azadirachtin that are derived only from plants. Insect pheromones are also

discussed in this chapter, although in a later subsection on "Insect Control Agents of Insect and Microbial Origin" there is a chapter devoted specifically to pheromones and related compounds.

Many of the errors occur in the chemical structures and reaction schemes depicted in the book. Some probably arise because of the generally poor and inconsistent way in which structures are presented, but others are more fundamental and are often accompanied by errors in nomenclature or by incorrect statements in the text. For example, in the opening part of the chapter on lindane, where nomenclature is discussed, the chemical structure of hexachlorobenzene appears with the name benzene hexachloride. The author reiterates this mistake in the text and adds to it by joining the words benzene and hexachloride. Another example may be

taken from the chapter on organophosphorus insecticides, in which, although the author makes two attempts to explain the nomenclature of sulphur containing organophosphorus compounds, the structures of the two forms of demeton, incorrectly named mercaptophos, are presented with the terms thiolo and thiono wrongly assigned. Several related mistakes then follow.

On many occasions in the book there appear statements that are misleading or obscure. Also, the selection of material often seems to be arbitrary and there is perhaps too much reliance on early literature. In the light of these impressions and the points exemplified earlier I cannot recommend this book.

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Biochemical taxonomy

Biochemical Phylogeny of the Protists. By M. A. Ragan and D. J. Chapman. Pp. 317. (Academic: New York, San Francisco and London, 1978.) \$29.50; £20.95.

IN 1866 Haeckel drew the first phylogenetic tree with three main branches: Plantae, Animalia and Protista. The last purported to show the taxonomic relationships between a diverse group of simple life forms which included the bacteria, fungi, protozoa, sponges and some algae. It subsequently became necessary to divide this group and retain the title of 'protista' for eukaryotic forms only; the prokaryotes, bacteria and blue-green algae, have been separated off as the 'monera' or 'lower protista'. Support for this, and other modifications to earlier phylogenetic trees, has come increasingly from chemical studies in addition to the morphological studies (that is, classical taxonomy). The aim of the present work has been to redraw Haeckel's original scheme using biochemical data, thus significantly extending the chemical approach to this subject.

Evolution has become a favourite topic amongst biochemists, as molecular studies have increasingly revealed the structure of homologous proteins from different organisms, for example, cytochrome *c*. The authors of this book, however, have not confined themselves to an examination of proteins alone; they also include a chapter on nucleic acids, another on metabolic pathways, four on metabolites, and one which is a miscellany covering diverse topics

such as sulphate reduction and mineral nutrition. Apart from possible contributions from the field of immunology, no area seems to have been left unexplored; and in parts the text inevitably becomes a catalogue listing similarities amongst organisms, albeit a useful reference for biochemical research.

The resulting phylogenetic tree bears a considerable resemblance to some other modern 'classical' trees. This is somewhat surprising as only 1 or 2% of the species within this group has been examined biochemically, even for widely used characters like pigmentation; and fewer still have been studied as representative species for taxonomic purposes. The results suggest, however, that the biochemical approach to taxonomy is at least as good as any other at present and that it promises well for the future because of its more fundamental nature.

The most readable part of the text (not withstanding such modern idiom as "upcoming pages") includes the initial chapters on phylogeny and evolutionary theory and the concluding chapters comparing phylogenies and summarising the role that biochemistry can play in this field. In addition to references, the systematic classifications and taxonomic index of relevant organisms, a glossary for the non-specialist, explaining such terms as 'semantide' and 'cladist', would have been welcome. Also welcome would have been some detail on the mechanics of tree construction, particularly from biochemical data; was, for example, a computer used?

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Celestial mechanics

Orbital Motion. By A. E. Roy. Pp.89. (Adam Hilger: London, 1978.) £12.50.

THE number of textbooks on celestial mechanics currently in print can be easily counted on the fingers of one hand. Professor Roy's excellent book is therefore very welcome. It provides a valuable bonus too, for, apart from the familiar topics which are to be found in other textbooks, *Orbital Motion* contains much other fascinating material. Though intended primarily for advanced undergraduate and postgraduate students, a good deal of the material could usefully be included in first and second-year undergraduate courses in applied mathematics and astronomy. Apart from a basic understanding of calculus and vector algebra, the reader requires little other mathematical knowledge.

There are fifteen chapters, each having a very good introduction to the subject matter and in most cases references to background and other pertinent material. The first chapter describes the various orbital phenomena which are observed in the Solar System and the more distant parts of the Universe. Coordinate and time-keeping systems and the reduction of observational data take up the next two chapters. There follow four chapters in which are developed the basic mathematical principles of celestial mechanics.

I like Professor Roy's treatments of the two and n-body problems, but I prefer some other derivations of Lagrange's solutions of the three-body problem. The restricted three-body problem is nicely handled, and an informative introduction to periodic orbits is provided. Lagrange's equations of planetary motion are derived in a standard way, but Gauss's form of the equations is stated without proof. This is a pity, for Gauss's equations are more general and their construction from first principles is instructive.

There is a very brief discussion of Hamiltonian systems, but the opportunity is missed to introduce the student to the widely used canonical variables of Delaunay. An ample and clear account is given of special perturbation techniques.

Chapter eight is devoted to a discussion of the evolution and stability of the solar system. The abundance of commensurabilities in mean motions is remarked on and possible reasons for their occurrence put forward. Many useful references are given here, though others are conspicuous by their absence (for example, papers by

A. T. Sinclair and by S. F. Dermott).

Elementary lunar and artificial satellite theories are developed next and the principal features of the motions described. I believe it must be an oversight on the author's part that Vinti's pioneering work in artificial satellite theory is not even cited in the bibliography.

There follow three interesting chapters concerning rocket and space-probe dynamics, in which the theories of orbit transfer, orbit determination and space navigation are clearly presented. The motions of binary and many-body stellar systems are investigated in the concluding chapters.

The student reader should be warned that several errors occur in the text; fortunately most of these are very minor. An error of a more serious nature occurs on p.174 where the author suggests that Lagrange's planetary equations and an appropriate

disturbing function R can be used to investigate the problem of atmospheric drag in artificial satellite theory.

Although the diagrams and tables are clear and informative, I feel that their usefulness would be enhanced if captions were provided. Many problems are listed, ranging from trivial to difficult, and their solutions, with hints if appropriate, are given at the end of the book. The index is comprehensive, and much useful data are assembled in several appendices.

The criticisms I have made hardly detract from the great value of the book. Professor Roy's style is fresh and pleasing to read and the overall presentation is of a high standard. I shall certainly be recommending the book to my students.

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Limb development

Vertebrate Limb and Somite Morphogenesis. Edited by D. A. Ede, J. R. Hinchcliffe and M. Balls. Pp. 498. (Cambridge University Press: Cambridge, London and New York, 1978.) £20.

THIS book, which comprises papers given at the third symposium meeting of the British Society for Developmental Biology in 1976, should interest all who are concerned with pattern formation in development and the problems of polarity determination. The volume, which is attractively presented, contains 29 papers: 5 on somitogenesis and 24 on aspects of limb development and regeneration.

In a short review it is not possible to comment on all the papers in this volume. The book is well represented, however, by the first contribution, which is a review article by Saunders. This is admirably clear and introduces the reader to the zone of polarising activity (ZPA) and to the apical ectodermal ridge, about which there has been so much fuss recently. Indeed, one of the surprises of the meeting was the comment, by Saunders, that polarising activity can no longer be regarded as exclusive to the ZPA.

It is refreshing at a time when approaches to development are largely molecular and biochemical to come across work which utilises so extensively the classical methods of experimental embryology. Many of the series of experiments reported here involve transplantations, deletions, reconstitutions, and so on, requiring great skill and, more often than not,

using the chick limb bud as the object of investigation. The questions being studied are basic to an understanding of animal development, such as the nature of, and the mechanisms underlying, axial polarisation, handedness, symmetry, reduplication, and so on. These questions are older than the science of experimental embryology; and though we must admit that (almost) none of the answers are known, this book does a very good job of reviewing the field and bringing the reader up to date in these complex matters.

The study of limb development and regeneration seems to have reached a stage when serious hypotheses can be proposed. This is suggested by the profusion of model-making activity here demonstrated. Models of limb development (and regeneration) discussed in this book include those of Wolpert, Summerbell and Tickle, Wilby, and Bryant. The competition to produce the most helpful model seems to be fierce but interactions between the various protagonists are friendly and positive. Such diverse activity can only help towards the understanding of the systems.

The chapters are in general clearly written and comprehensible. The line drawings are excellent, and the half-tones are acceptable (barely). The volume has a good subject index but unfortunately no general index of authors cited. Even so, the book can be strongly recommended to all interested in pattern formation and the determination of polarity.

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